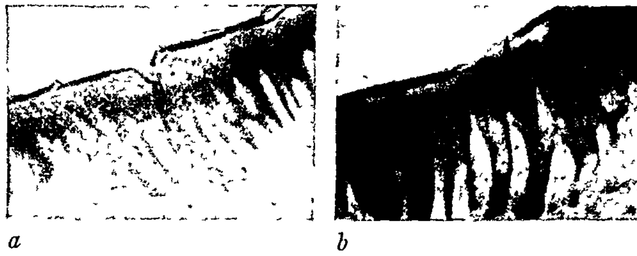


FIGURE 1 (*a*) Succinic dehydrogenase plus endogenous activities in the absence of cyanide ×100 (*b*) Succinic dehydrogenase plus endogenous activities in the presence of cyanide ×100 See Bertram Eichel "Oxidative Enzymes of Gingiva," page 479

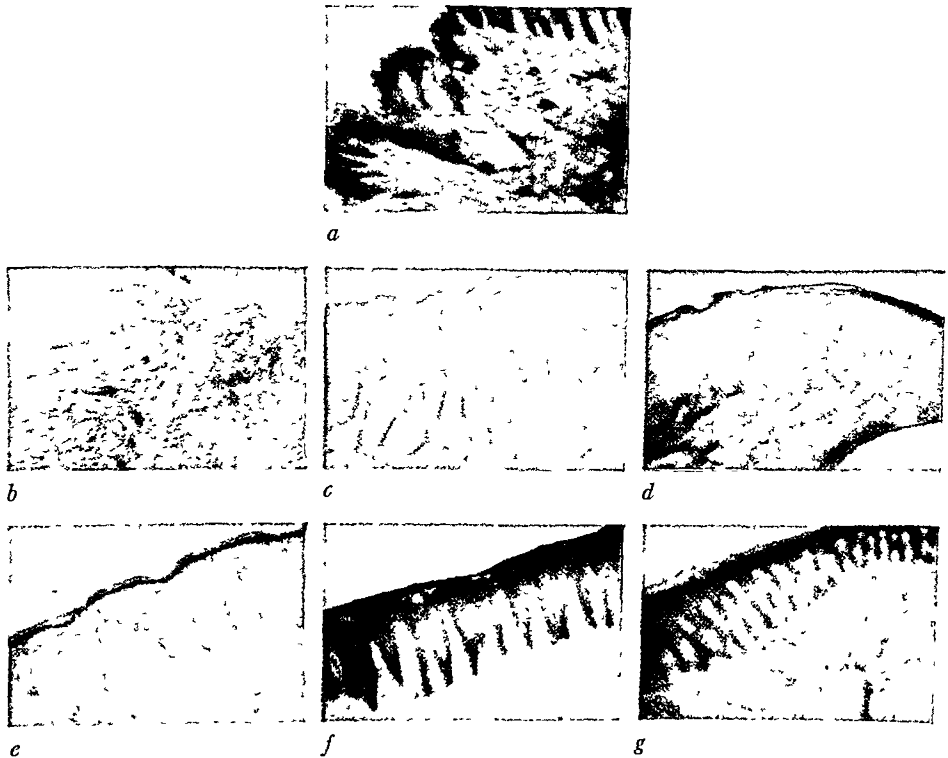


FIGURE 1 (*a*) Alkaline phosphatase ×100. (*b*) Beta-D-glucuronidase ×100 (*c*) Galactosidase ×100 (*d*) Glucosidase ×50 (*e*) Total esterases. ×100 (*f*) Acid phosphatase ×100 (*g*) Cholinesterase (pseudo) ×100 See Vincent F Lisanti, "Hydrolytic Enzymes in Periodontal Tissues," page 461.

(*Frontispiece*)

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THE METABOLISM OF ORAL TISSUES*

Conference Chairman and Consulting Editor

PHILIP PERSON

CONTENTS

* This series of papers is the result of a conference on *The Metabolism of Oral Tissues* held by The New York Academy of Sciences on October 15, 16, and 17, 1959. Publication of this monograph was supported in part by Research Grant D-1138 from the National Institute of Dental Research, Public Health Service, Bethesda, Md

Part III. The Salivary Glands

Part IV. The Tongue and Oral Mucosa

Part V. The Periodontal Tissues



JOSEPH GOLDBERGER
(1874 to 1929)

(Photograph courtesy of William H. Sebrell)

DEDICATION TO JOSEPH GOLDBERGER

(1874 to 1929)

By Philip Person

Veterans Administration Hospital, Brooklyn, N. Y., and Rutgers University, New Brunswick, N.J.

This monograph is dedicated to the memory of Joseph Goldberger. In the course of a brilliant career, during which he made many contributions, he pioneered in the study of oral tissue metabolism. His life and work have been an inspiration to many individuals.

Joseph Goldberger and his associates in the United States Public Health Service made known the cause of pellagra, an epidemic disease, and methods for its control. From their studies there also came a greater knowledge and awareness of interrelationships between changes in oral tissues and the nutritional and metabolic status of the organism. These brought with them an increased realization of the importance of a careful examination of the oral tissues as part of the physical examination of every patient. It is difficult for some who take this knowledge for granted to realize that, as a result of this pioneering work, major medical and sociological advances have occurred whose ends are not yet in sight. At the conference on The Metabolism of Oral Tissues, upon which this monograph is based, we were reminded by William H. Sebrell of Columbia University, New York, N. Y., who was one of Joseph Goldberger's associates, that in spite of the remarkable achievements referred to above, the intermediary biochemical and metabolic processes responsible for the oral tissue changes in diseases of nutrition and metabolism are still largely unknown. It is hoped that this monograph will stimulate research that will lead to some of the answers to these problems.

The conference on The Metabolism of Oral Tissues was in many ways the first of its kind. Neither the conference nor this monograph would have been complete without reference to, and recognition of, the monumental contributions to the public health made by Joseph Goldberger and his associates.

INTRODUCTION: A STEPPING STONE TOWARD DENTAL HEALTH

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At a joint meeting of the International Association for Dental Research, Chicago, Ill., and the American Association of Dental Schools, Minneapolis, Minn., held in 1957 at Detroit, Mich.,[1] I reached the conclusion that recent progress in dental research is one of the best-kept secrets in modern science. I made that comment in view of the considerable discrepancy between the traditionally narrow concept of dentistry and the broadening spectrum of dental science.

This year, as organized dentistry in the United States is celebrating its first centennial, we can look back with pride at a century of significant progress in the art and science of dentistry. Through contributions ranging from ether inhalation to water fluoridation, the goals of dentistry have moved away from simple extraction and repair to control and prevention of oral disease. This blending of art and science has given a broader professional content both to the education of students and to the care of patients.

Dental science already has a seemingly enormous advantage over the recent past. A generation ago only a few schools were engaged in scholarly pursuits. In 1926 in the United States all dental schools together spent less than $50,000 for research. Today the majority of the nation's 47 dental schools conduct research. Scholarly activities have become a normal day-to-day activity in academic dentistry, and almost as many other scientific institutions (state, private, and industrial) are concerning themselves with some of the aspects related to oral health and disease. However, we cannot disregard the fact that we are still baffled by several destructive and disfiguring oral diseases because we know far too little about the deeper secrets of the metabolism of oral tissues.

A case in point is periodontal disease, characterized by inflamed and receding gingivae, rapid destruction of alveolar bone, and resultant loss of teeth, a widespread problem in our aging population. Possibly some aspects of dental research represent a more difficult field of science than was previously believed. Based on the analogy of so many other developmental experiences in health and welfare, it is reasonable to assume that the answers to fundamental questions concerning oral lesions will be interrelated, not only among themselves but also with the answers to problems in biology of more general nature

Today, a new strain of "oral biologists" is being groomed through various programs; some schools emphasize laboratory sciences, others concentrate on clinical specialty training. These various methods of advanced postdoctoral education for dentists represent one of the United State's most important recent contributions to modern dentistry. Broadly trained men are graduating from dental schools in increasing, although still inadequate, numbers to staff the dental faculties here and abroad, the research laboratories, the clinics, and private dental offices.

Five years ago The New York Academy of Sciences published *Recent Advances in the Study of the Structure, Composition, and Growth of Mineralized Tissues*,[2] a monograph that undoubtedly has had a stimulating effect not only on dental research, but on the general topic of calcification in biological systems. Since then we have witnessed a continuing interest in this area, as indicated by the annual Gordon Research Conferences on Bones and Teeth in the United States and the organization of the Bone and Tooth Society of Great Britain; last year the Section on Dentistry of the American Association for the Advancement of Science organized a multidisciplinary symposium on calcification in biological systems,[3] which covered the entire animal kingdom, from lobster claws to human teeth.

This monograph attempts to review current knowledge of the mouth over and beyond the dental apparatus and to cover the entire problem of the development, structure, environment, and metabolism of the tissues in the oral cavity. Included are the evolutionary aspects of these organs that have contributed so much to man's life, livelihood, and culture, and various extraneous and internal influences, operating from prenatal life to maturity, that modify the health and welfare of the oral tissues. Particularly timely is the inclusion in this publication of an up-to-date consideration of the soft tissues of the mouth. As the life span increases, diseases in these structures are assuming increasing significance from the point of view of development and maintenance. Even under normal circumstances, the very process of aging may well be better understood as new approaches with newer tools of science are brought to bear on the study of biological changes in the mucous membrane of the mouth.

This monograph examines all of the principal tissues that are critically involved in oral pathology, including, specifically, the hard tissues of the teeth, the enamel and dentin, which are involved in tooth decay, the alveolar bone and other supporting tissues of the teeth involved in periodontal disease and malocclusion; the oral mucous membrane that well may prove to mirror nutritional, hormonal, and other metabolic disturbances and a variety of atrophic, hypertrophic, and neoplastic pathology; and, finally, the salivary gland structure and function related to the maintenance of health, both in the hard and soft tissues of the mouth.

In terms of general principles, it is evident, therefore, that this monograph spans several of the most important biological mechanisms that are basic to oral health. namely, calcification, cornification, secretion, and host-parasite relationship. In this scientific context the subject at hand is not only of singular importance to oral health, but is of general interest to biological science and will contribute to the pool of fundamental knowledge from which we all draw.

The scientific evolution underlying research presented in this monograph was brought about by scientists in many fields, inside and outside of dentistry and before the present era, including Jacob Erdheim[4] of Austria who, in 1911, as a result of his hormonal studies likened the tooth organ to the drum of a metabolic kymograph; May and Edward Mellanby of England, who showed the sensitivity of the bone and tooth substance to abnormalities in the calcifying property of the diet with special reference to vitamin D; György Hevesy of

Denmark who, in the first application of man-made radioisotopes to biological research in 1935, brought the dental hard tissues into the orbit of ionic exchange mechanisms; William Gies, founder of the International Association for Dental Research and its *Journal of Dental Research*; William K. Gregory, whose elegant studies of dental evolution lent stature to the first issues of the *Journal*, and S. Burt Wolbach and Percy R. Howe, pathologist and dentist respectively, who collaborated in pioneer studies of cellular performance of dental tissues in response to accessory food factors at a time before these were known as vitamins.

Today dentistry not only strives to know expertly *how*, but also to understand deeply *why*. Further steps must be taken to implement the education of more dental scientists; to make it possible for properly trained dental investigators to pursue basic research on a long-term basis rather than remain confined to narrow, short-term projects; and to facilitate opportunities for this new breed of dental scholars and investigators to extend and deepen their collaboration with scientists and experts in other fields, ranging from the laboratory sciences to chairside and bedside patient care. Hopefully, this three-pronged approach will elevate the entire academic triad of teaching, research, and service for dental health. These goals are now attainable. Continued ignorance, to paraphrase Alfred North Whitehead, has the guilt of vice, when attainable knowledge can change the course of events. I am confident that dentistry will face the next century with a new perspective, knowing more about the relation of the parts to one another and to the whole, and extending the vista of our research toward ultimate solutions of oral health problems.

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Part I. Perspectives on Oral Tissue Metabolism

SOME OBSERVATIONS ON THE EVOLUTION OF ORAL TISSUES

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Introduction

Since we are currently celebrating the centennial of Charles R. Darwin's *The Origin of Species* it seems especially appropriate to begin a publication such as this, in which so many disciplines of biology are represented, upon a theme of evolution.

Perhaps no vertebrate structures have received more detailed evolutionary study than have teeth and jaws. Even so, few who work with oral tissues are aware of their importance in some of the great problems of biological evolution. Neither is it generally appreciated that there is much in the story of the evolution of oral structures that can improve our understanding of, and approach to, problems of oral tissue metabolism. Some preliminary remarks concerning these matters are therefore in order.

Nearly all the great evolutionary advances have involved major changes in food-getting. Edwin H. Colbert has said that in the course of vertebrate evolution the appearance of jaws constituted a major revolution whose importance cannot be overestimated.[1] Why this should be so may be easily understood from the following brief passage taken from Homer W. Smith's *From Fish to Philosopher:*

"Without the predatory power of jaws and teeth and the possibility of swift and accurate pursuit of prey there would have been no evolution of the distance-sense organs of smell, sight and hearing, of elaborate muscular co-ordination, of prevision of how to get from here to there and the possible consequences of the transit—in short, there would have been no centralization of the nervous system such as ultimately produced the brain, and the earth would never have known the phenomenon of consciousness, at least of an order superior to that of the lobster, scorpion, or butterfly."*

Another reason, readily appreciated, for the very great importance of the teeth and jaws in evolutionary studies is their relative indestructibility. This is especially the case in respect to problems of the origin of man. Quite frequently the remains of man, apes, monkeys, and related mammals consist solely of broken jaws with a few teeth. These structures therefore may constitute the only evidence from which anthropologists and paleontologists must evaluate the hominid character of such remains. For this reason the morphologies of the jaws and teeth have become reference criteria in investigations of the origin of man as well as of other problems of evolution.[3,4]

It is not generally known that another major reason for the critical impor-

* Quoted by permission of Little, Brown & Company, Boston, Mass [2]

tance of the teeth in our knowledge of vertebrate evolution is that their study produced what Henry Fairfield Osborn called one of the greatest generalizations ever made in mammalian comparative anatomy. This was the theory of the trituberculur origin of the molar, first set forth by E. D. Cope in 1888[5] and later modified by Osborn.[6] The essence of this theory is that a three-cusped tooth occurring in lower mammals served as the prototype of the molar teeth found in higher mammals. Tracing the vissicitudes of these three cusps through the various mammalian groups, Cope recognized that they provided a fixed frame of reference that transformed static tooth morphology into an excitingly dynamic analytic tool of evolutionary study.

Mastication, a function that most of us today take for granted, thanks to the ingenious restorative methods developed by modern dentistry, is a matter of life and death for most other vertebrates. Because of this the development of the teeth and jaws has, in the course of evolution, often marked a species for survival or extinction. This has been especially well documented in the case of the horse. In his wonderful book on horses, George Gaylord Simpson[7] dramatically portrays how in the evolution of the horse a major shift occurred: from browsing on soft leaves to grazing on hard grasses Concomitantly, the evolution of the molar tooth resulted in the formation of thicker, longer crowns, whose enamel crevices were filled with a hard cement. Such teeth could withstand the tremendous abrasive action of grinding hard grasses. However, when molars did not change in this fashion, the teeth rapidly wore down, and horses with worn teeth were doomed to extinction because they could not gather and chew their food.[8]

Before going further, I must acknowledge that, with respect to a critical knowledge of the problems of evolution mentioned above, I qualify only as an "interested layman." My audacity in attempting this presentation stems from the fact that, although comparative morphology, paleontology, and anthropology have created a fascinating picture of the evolution of the gross morphology of oral structures, there have been surprisingly few attempts to gain insight into problems of the evolution of oral structures at cellular and tissue levels, or to understand how evolutionary factors and forces influence, or may be involved in, problems of oral tissue physiology and biochemistry.

The evolutionary approach to physiology and biochemistry is not a common one but, when used, has often brought new summits of understanding. For example, we cannot conceive of our present knowledge of renal physiology[9,10] or of neuroanatomy and neurophysiology,[11,12] or of the biochemistry of vision[13,14] without acknowledging our tremendous indebtedness to comparative and evolutionary studies. In the remainder of this presentation, therefore, an attempt will be made at an evolutionary approach to some problems of oral tissue metabolism.

The Origins of Enamel, Dentine, Bone, and Cartilage

Enamel, dentine, and bone originated together more than 400 million years ago. They were all components of the plates of bony armor that covered the primitive, jawless vertebrates known as ostracoderms. These animals, the oldest and most primitive vertebrates, were small (averaging between 6 and 12 inches in length), sluggishly moving "self-propelled food traps,"[15] with

"vacuum-cleaner" mouths[16] that sucked in everything within range; they used gills as strainers. In the most primitive ostracoderms osteocytes were not found. A cross section[17] of the epidermal armor plate of one of these animals reveals an outer layer of a glassy, enamel-like substance that rested on a dentinelike material, cosmin. The latter was in turn supported by isopedin, a bonelike material which, however, did not contain true bone cells. The tissues mentioned above were laid down appositionally in successive or concentric layers. Running through the basal and mid regions of the armor were vascular channels, continuous with more superficial, radiating, and interconnecting mucous canals, which opened to the exterior. In later ostracoderms the most significant evolutionary change was that the noncellular isopedin was replaced by a bone tissue built by osteoblasts and containing osteocytes.[18] Here, too, radiating and interconnecting mucous canals opened to the exterior. Two items engage our interest at this point. The first is that enamel, dentine, and bone first appeared in nature together as parts of an epidermal structural complex. The second is that all these tissues, especially dentine and bone, originated in proximity with mucous canals. One may therefore consider that mucopolysaccharides and mucoproteins, which today are recognized as important components of these tissues, may have been constituents of the fluids in which vertebrate calcifying cells first assumed their character and functions.

It has been suggested that the development of the ostracoderm's bony armor constituted a means of defense against large and vicious predators that lurked in the environment.[19] However, Homer Smith has proposed an intriguing alternate explanation.[20] Based upon the contention that ostracoderms, which lived in fresh water, took origin from sea- or saline-dwelling ancestors, Smith defined the "death-dealing enemy" of the ostracoderms, "swift, merciless, and irresistible," not as an animal but as a physicochemical enemy, namely, the entrance of saline-rich tissues into fresh water. He suggested that the function of the armor was to keep water out of the body. Without the protection of an outer waterproof armor, osmotic water movement into the body would have caused death by "dissolution as a gelatinous mass." This was one of the first broad intimations that bone was a factor in the regulation of the internal environment of the body. At the time that this proposal was made, biochemists and physiologists knew that bone was an important factor in the regulation of Ca^{++} levels in body fluids. They did not know something that is only now being realized: that bone also participates in the regulation of levels of other ionic components of body and tissue fluids,[21,22] such as Mg^{++}, Na^{+}, and K^{+} (and possibly of organic compounds as well). Thus today, more than twenty years later, Smith's proposal is finding support and extension in critical biochemical and physiological experiments that are gaining recognition for bone as a major participant in the regulation and maintenance of the constancy of the internal environment of the body.

Let us turn now to a consideration of the origin of cartilage. It would appear that at some point in vertebrate evolution cartilage and bone, like Ruth and Naomi, decided "Whither thou goest, I will go" (with exceptions, of course). This has led to the belief that both tissues originated in the ostracoderms.[23] However, it should be noted that direct evidence for this has never been found. It is reasoned that cartilage was a device developed in the ostra-

coderm embryo for providing a pliable endoskeleton capable of better accommodation to early, rapid growth and developmental changes than bone. It has also been assumed that, being softer than bone, the cartilage tissues were not preserved after death and therefore could not be fossilized. This conjecture may be valid, but that cartilage as a tissue type originated in the ostracoderms is open to serious question. For as a tissue type cartilage first appeared in animals without backbones, the invertebrates,[24,25,26] before ostracoderms appeared on the evolutionary scene. Some examples of invertebrate cartilages may be seen in FIGURE 1*a*,*b*, and *d*, which show the histological appearance of tissues from two molluscs, *Busycon canaliculatum*, the whelk, and *Loligo pealii*, the squid, and also from an arthropod, *Limulus polyphemus*, the horseshoe crab. These cartilages are all endoskeletal.[27,28,29] Most striking is the fact that the *Busycon* cartilage contains myoglobin.[30,31] It will be seen that the whelk and horseshoe crab tissues (FIGURE 1*a* and *d*) are characterized by relatively scant matrix in relation to cell content. The squid cartilage (FIGURE 1*b*), on the other hand, contains greater amounts of matrix and appears similar to some types of vertebrate hyaline cartilage. At first glance the scant matrix cartilages (FIGURE 1*a* and *d*) seem quite different from vertebrate cartilages, but FIGURE 1*c*, a photomicrograph of xiphisternal cartilage from the rat, shows that striking similarities exist between the invertebrate and vertebrate tissues with respect to matrix relationships. A similar type of scant matrix tissue is found in mammalian limb bud cartilage, seen in very young embryos in whom the cartilage is just taking origin from its mesenchymal field.[32] Thus, in some respects, under light microscopy the vertebrate and invertebrate cartilage tissues are quite similar, although very significant differences also exist. It should be mentioned, in addition, that the invertebrate cartilages contain chitin,[33] do not yield gelatin when boiled,[31] and do not contain chondroitin sulfate[30,35]

Darwin's concept of evolution is not the present concept of evolution. It is no longer believed that a single continuous chain of development leads from the simplest to the most complex organisms. It is a cardinal principle of zoology that invertebrate structures are never precursors or homologues of vertebrate structures Even when striking morphologic resemblances such as we have just seen are found, they are never considered part of a continuous evolutionary process but rather examples of parallel but unrelated evolutions. Thus, it was decided about forty years ago that the invertebrate cartilage tissues were not cartilaginous but chondroid[25] Since that time, and because of this decision, the tissues have been almost completely overlooked and unstudied.

That there may be morphologic reasons for deciding that invertebrate cartilaginous tissues did not evolve from vertebrate cartilage and therefore are unimportant for an understanding of vertebrate cartilage cannot be denied However, during the past forty years some biochemical doctrines have developed indicating that this invertebrate cartilage or chondroid may indeed be very important for an understanding of the biochemistry and metabolism of vertebrate cartilage It is a cardinal principle of modern biochemistry that certain fundamental metabolic processes are ubiquitous in nature Thus glycolysis, respiration, fatty acid oxidations, and proteolysis, as well as more com-

plex processes involving integrations at higher levels, have been shown to occur in nearly all animal and plant cells. Therefore, the biochemist does not hesitate to homologize at chemical and metabolic levels between all forms of living

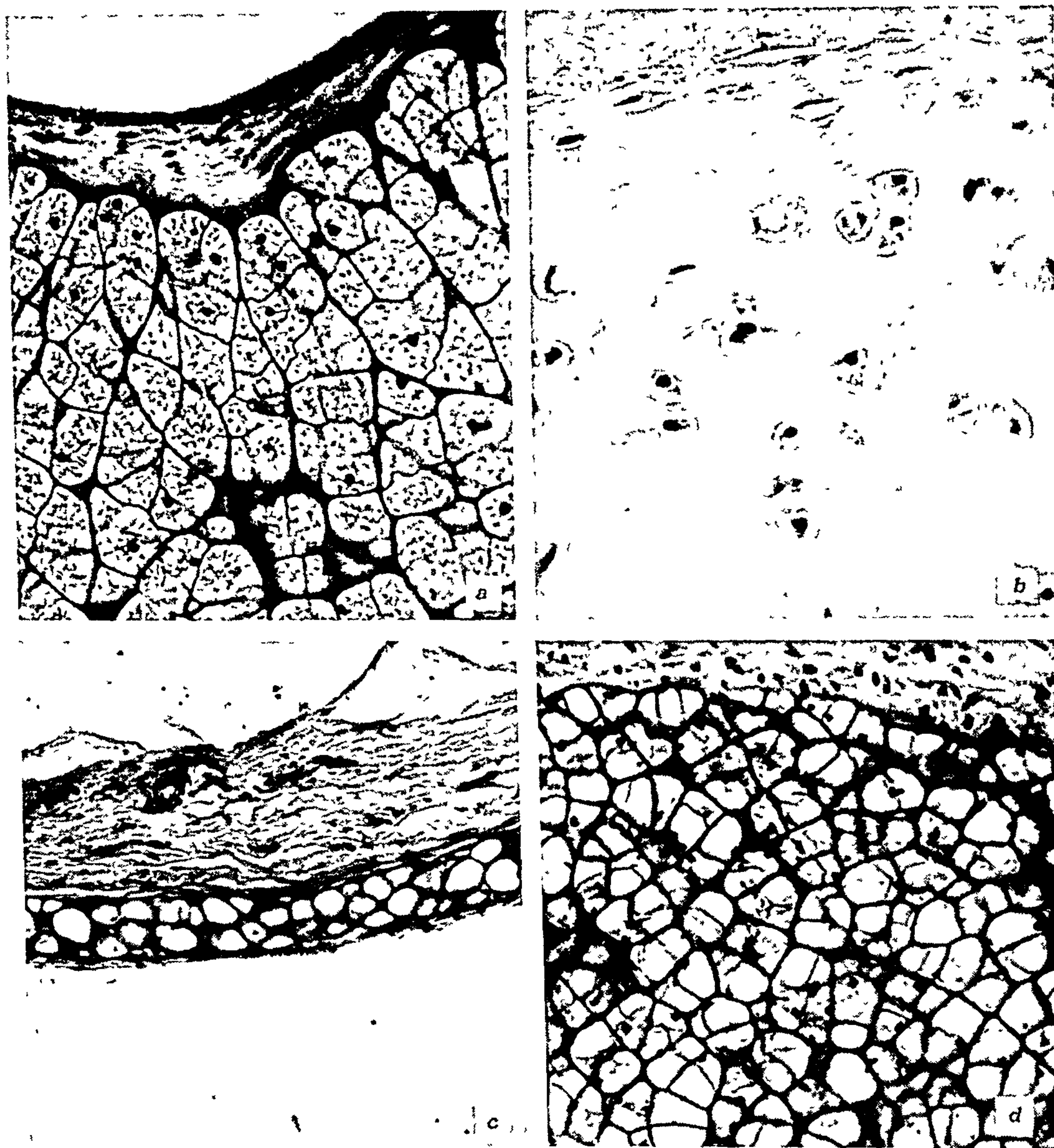


FIGURE 1 Sections of cartilage tissues. Hematoxylin and eosin stain Original magnifications, ×140 (*a*) Odontophore cartilage from the marine snail, *Busycon canaliculatum* (*b*) Head cartilage from the squid, *Loligo pealii* (*c*) Xiphisternal cartilage from the rat (*d*) Branchial cartilage from the horseshoe crab, *Limulus polyphemus*

matter. Moreover, since structure and function are but different sides of the same coin, one may at least suspect that, where there are striking similarities in structure, there are fundamental physiological, biochemical, and metabolic similarities An analogous situation may be found in the evolution of vision. George Wald has pointed out that three separate times in the course of evolution Nature has created eyes.[14] In all the animal phyla eyes are found only

coderm embryo for providing a pliable endoskeleton capable of better accommodation to early, rapid growth and developmental changes than bone. It has also been assumed that, being softer than bone, the cartilage tissues were not preserved after death and therefore could not be fossilized. This conjecture may be valid, but that cartilage as a tissue type originated in the ostracoderms is open to serious question. For as a tissue type cartilage first appeared in animals without backbones, the invertebrates,[24,25,26] before ostracoderms appeared on the evolutionary scene. Some examples of invertebrate cartilages may be seen in FIGURE 1*a*,*b*, and *d*, which show the histological appearance of tissues from two molluscs, *Busycon canaliculatum*, the whelk, and *Loligo pealii*, the squid, and also from an arthropod, *Limulus polyphemus*, the horseshoe crab. These cartilages are all endoskeletal.[27,28,29] Most striking is the fact that the *Busycon* cartilage contains myoglobin[30,31] It will be seen that the whelk and horseshoe crab tissues (FIGURE 1*a* and *d*) are characterized by relatively scant matrix in relation to cell content. The squid cartilage (FIGURE 1*b*), on the other hand, contains greater amounts of matrix and appears similar to some types of vertebrate hyaline cartilage. At first glance the scant matrix cartilages (FIGURE 1*a* and *d*) seem quite different from vertebrate cartilages, but FIGURE 1*c*, a photomicrograph of xiphisternal cartilage from the rat, shows that striking similarities exist between the invertebrate and vertebrate tissues with respect to matrix relationships. A similar type of scant matrix tissue is found in mammalian limb bud cartilage, seen in very young embryos in whom the cartilage is just taking origin from its mesenchymal field[32] Thus, in some respects, under light microscopy the vertebrate and invertebrate cartilage tissues are quite similar, although very significant differences also exist. It should be mentioned, in addition, that the invertebrate cartilages contain chitin,[33] do not yield gelatin when boiled,[34] and do not contain chondroitin sulfate.[30,35]

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draw from their surroundings certain substances, and they were further endowed with the capacity to synthesize from these substances the tissue known as bone. To understand how this was done, we must study these processes as they emerged in natural history. This approach has one virtue, if no other. It focuses attention squarely on the core problem, namely, how do cells form bone? Going further, we are made aware that there are primary processes of bone formation concerning which we know very little, and secondary processes concerning which we know much more. The primary processes are in the realm of cell and tissue metabolism, for osteoblasts are cells but in three dimensions they also form tissues; the secondary processes are such effects as those of hormones and muscle function, which usually act as regulators of the rate of the primary processes occurring within the osteoblasts. To put the matter in this way is not to say that the secondary factors are not extremely important. It should be remembered, however, that secondary problems always gain and never lose when the primary problem is kept in mind. Another way of saying this is that it was a good thing that when Luigi Galvani discovered the electric current he was interested in frog muscle and not trolley cars.[39]

In closing, it would be negligent not to mention a fact that is not directly related to the topic of this monograph, but is perhaps the most exciting development in the field of evolution since Darwin's original thesis. This is the clarification of the chemical structure of nucleic acids,[40] which now makes possible the creation of a biochemistry of genetics.[41] As a result, we stand today on the threshold of an understanding of what has been called "the molecular basis of evolution."[42] It is only within the past decade that such a phrase could be formulated in a meaningful way; its full meaning cannot yet be grasped. Still, there could be no better way of closing a talk on evolution a century after Darwin than by saluting a new discipline that will undoubtedly carry us to greater understanding of the mysteries first unfolded in *The Origin of Species*.

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in molluscs, arthropods, and vertebrates. These eyes do not represent a continuous evolutionary process. They also differ anatomically and embryologically. However, here is the striking fact: the fundamental biochemical basis of vision is very nearly the same in all three types of eye. It is the absorption of light by vitamin A aldehyde joined to the class of retinal proteins known as opsonins.

It is being argued that decisions made earlier, to the effect that invertebrate cartilages are less important or that they are not cartilage, based as they are upon morphologic criteria of almost half a century ago, are no longer acceptable. It is contended here that until more is known about the similarities and differences between vertebrate cartilages and invertebrate cartilages and about their definitive biochemical and metabolic characteristics, it is preferable not to read the invertebrate tissues out of the cartilage family, as apparently has been done.

There is still another reason why the invertebrate cartilages are of great importance. They are a magnificent experiment in nature. Unlike vertebrate cartilage, these tissues will never participate in bone formation. May it not be that significant biochemical, metabolic, and morphologic factors exist that can explain why one tissue does, and the other does not, become involved with hydroxyapatite crystals? Such considerations apply, of course, not only to invertebrate cartilage tissues, but to the invertebrate calcified tissues, which also do not form hydroxyapatite. In this connection there is a very interesting report by Buffa[36] to the effect that invertebrate calcified tissues contain only minute and insignificant amounts of citrate, whereas vertebrate calcified tissues, as we know, may have very significant amounts.

In recent work with the invertebrate cartilages it has been possible to demonstrate, for the first time in any cartilage tissue, the presence of typical aerobic respiration mediated via cytochrome oxidase activity.[37,38] The experience gained in this work has also permitted demonstration of this important terminal respiratory enzyme in vertebrate cartilage as well, a demonstration that had not been possible for thirty years. In very significant recent studies, Lash[30] and Lash and Whitehouse[35] have found that *Busycon* cartilage does not contain amino sugars, which are found in chondroitin. Instead, the invertebrate tissues contain glucose sulfate in a polymerized form and no chondroitin sulfate. When it is recalled that chitin contains polymerized glucosamine, the thought inevitably arises that there may have been a biochemical evolution of sugar derivatives as molecular components of animal skeletal tissues.

Summary

By analogy, the essence of this approach to the metabolism of oral tissues is that, as the study of the comparative morphology and mechanical functions of the vertebrate skeleton has provided a basis for comprehension of the morphology and function of the human skeleton, so we may expect that studies of the comparative biochemistry and physiology of oral structures will lead to a better understanding of their metabolism in the human Taking the example of bone in the above context, the central problem becomes this. in the course of evolution certain cells were endowed with the capacity to with-

draw from their surroundings certain substances, and they were further endowed with the capacity to synthesize from these substances the tissue known as bone. To understand how this was done, we must study these processes as they emerged in natural history. This approach has one virtue, if no other. It focuses attention squarely on the core problem, namely, how do cells form bone? Going further, we are made aware that there are primary processes of bone formation concerning which we know very little, and secondary processes concerning which we know much more. The primary processes are in the realm of cell and tissue metabolism, for osteoblasts are cells but in three dimensions they also form tissues; the secondary processes are such effects as those of hormones and muscle function, which usually act as regulators of the rate of the primary processes occurring within the osteoblasts. To put the matter in this way is not to say that the secondary factors are not extremely important. It should be remembered, however, that secondary problems always gain and never lose when the primary problem is kept in mind. Another way of saying this is that it was a good thing that when Luigi Galvani discovered the electric current he was interested in frog muscle and not trolley cars.[39]

In closing, it would be negligent not to mention a fact that is not directly related to the topic of this monograph, but is perhaps the most exciting development in the field of evolution since Darwin's original thesis. This is the clarification of the chemical structure of nucleic acids,[40] which now makes possible the creation of a biochemistry of genetics.[41] As a result, we stand today on the threshold of an understanding of what has been called "the molecular basis of evolution."[42] It is only within the past decade that such a phrase could be formulated in a meaningful way; its full meaning cannot yet be grasped. Still, there could be no better way of closing a talk on evolution a century after Darwin than by saluting a new discipline that will undoubtedly carry us to greater understanding of the mysteries first unfolded in *The Origin of Species.*

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ORAL STRUCTURES GENETICALLY AND ANTHROPOLOGICALLY CONSIDERED

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It is practically impossible to dichotomize this general review: what is heritable is frequently racial, and racial traits, in turn, are essentially genetic. Hence, no attempt will be made to present data in an exclusive genetic, or an exclusive anthropological, category. Instead, three major themes will be developed: first, structural (teeth, eruption sequence, dental arches, and jawbones); second, functional (faciodental growth and occlusion); and, finally, pathological (dental caries).

Teeth: Eruption Sequence, Dental Arches, Maxilla, and Mandible

Standard dental texts give detailed data on the dimensions of all of the teeth (*see* summary in Krogman, 1927). The size of the molar teeth has received the most frequent anthropological consideration. TABLE 1, taken from Stein and Epstein (1934), is an example of such studies. It is generally stated that the length of the molars, especially M1, is greatest in order of Negroid, Mongoloid, Caucasoid; the latter two are often reversible and are set apart from the first. Molar length, especially in the mandible, is in order of M1, M2, M3, M3 being extremely variable.

Sex differences in tooth size are not marked, although male teeth are usually somewhat larger than female (often noted in upper I1). For Melanesians, Stein (1936) offers the data in TABLE 2.

The inheritance of tooth size has been considered generally by Rushton (1953), who asserts that a large upper I1 is a dominant trait. Unilaterally large teeth, most frequently m2, C, and M1, are often associated with hemihypertrophy, although mode of inheritance is not clear. Very small, widely spaced teeth often accompany hereditary opalescent dentine and osteogenesis imperfecta. The most carefully controlled genetic study is that of Horowitz *et al.* (1958), who worked with 54 pairs of like-sexed Caucasian adult twins. These investigators concluded that "genetically conditioned variations of a highly significant nature occur in 8 of the 12 anterior teeth studied. The canine teeth demonstrate a relatively low hereditary component of variability." More specifically, it was concluded that "sex and asymmetrical factors appear to play a part in the variation observed in the maxillary left central, and the mandibular left canine and lateral teeth."

Hellman (1928) offers the best data on racial traits in the lower molar cusp patterns (FIGURE 1 and TABLE 3). The Y5 or "*Dryopithecus* pattern" is said to be ancestral to the Hominoidea. In this sense, the lower M1 retains, more than do M2 and M3, the more "primitive" cusp pattern (FIGURE 2, TABLE 3; also Dahlberg, 1945*a*).

A racial trait frequently studied is that of shovel-shaped incisors, so called because of an extra marginal enamel ridge on the lingual aspect of the teeth. It is most highly developed on the upper I1, so that the tooth, viewed lingually,

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The origin of the shovel-shaped trait is obscure. Abrahams (1949) speculates whether it is due to over-growth of the external or lateral growth loci in the tooth or to under-growth of the median locus. In a study of the peoples of Malayan origin residing in South Africa, Abrahams suggests that the trait is a recessive; moreover, since the Cape Malays show the trait less frequently than do the indigenous Malays, it is speculated that the former are evolving a "nor-

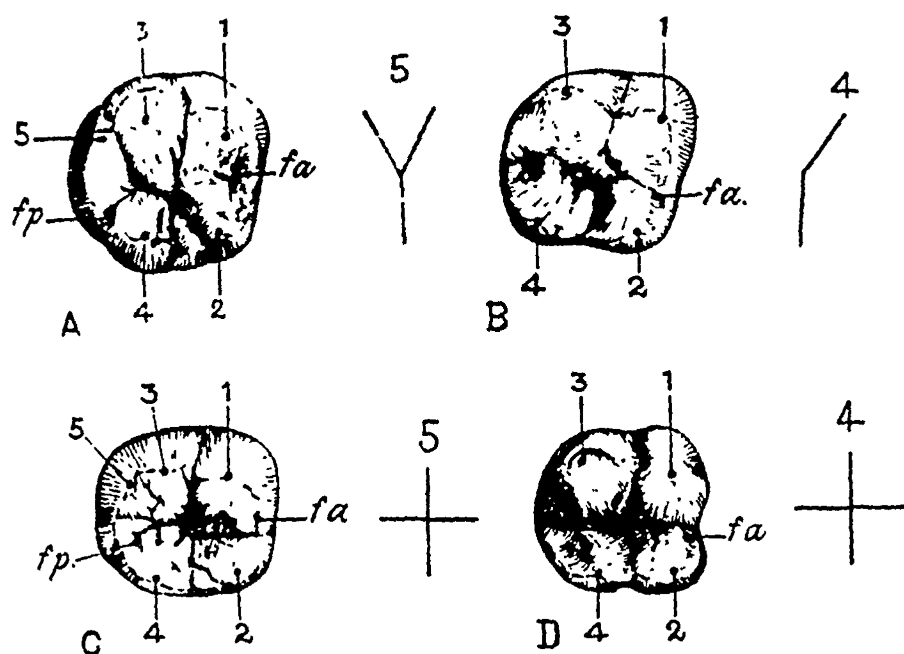


FIGURE 1 Four stages of development of lower molar teeth, illustrating the different changes from *Dryopithecus* to the most advanced pattern *A*, *Dryopithecus* pattern indicated by Y5, meaning primitive system of grooves and cusp formula; *B*, modified *Dryopithecus* pattern indicated by Y4, meaning cusp formula reduced to 4 and groove system primitive, with loss of the posterior limb of the Y, *C*, primitive cusp formula retained, but groove system changed to cruciform, the sign being +5, *D*, cusp formula and groove system changed, the sign being +4 Reproduced from Hellman (1928) by permission of *Proceedings of the American Philosophical Society*

TABLE 3
PERCENTAGE DISTRIBUTION OF CUSP PATTERNS OF M1, M2, AND M3*

	M1			M2			M3		
Sample	Y5	+5	+Y4	Y5	+5	+Y4	Y5	+5	+Y4
Chinese	100	—	—	—	19	81	—	50	50
Australian aborigine	100	—	—	5	43	52	14	72	14
American Indian	100	—	—	1	30	69	—	75	25
Asiatic Mongoloid	100	—	—	—	31	69	—	77	23
West African Negro	99	—	1	17	8	75	20	60	20
American Negro	98	1	1	6	24	70	4	63	33
Eskimo	97	3	1	6	37	57	—	52	48
American white male	87	2	11	—	1	99	4	34	62
Ancient European	83	—	17	3	11	86	6	34	60
	Sample size ranges 18–179			Sample size ranges 21–218			Sample size ranges 16–187		

* Reproduced from Dahlberg (1945) by permission of the *Journal of the American Dental Association*

resembles a small shovel. Often associated with this condition are labial lateral ridges, labial axial grooves, and rounding of the lateral incisors. Shovel-shaping, alone or in complex with the other traits, is said to be more frequent in the Mongoloid peoples of the world, although it is found among all peoples.

Hrdlicka (1920—see also Dahlberg, 1945) has summarized the racial incidence of shovel-shaped maxillary I1 and I2, as shown in TABLE 4.

TABLE 1
LENGTH (L) AND BREADTH (B) OF THE MOLAR TEETH IN MILLIMETERS*

	M1		M2		M3	
	Upper					
	L	B	L	B	L	B
Australian aborigine	11 4	12.8	10.9	13.1	10.0	12 3
Bantu	10.3	11.0	10.0	11.5	9.5	11.0
Bushman	9 9	10.6	9.7	10.6	8.2	10.3
American white	10 7	11.8	9 2	11.5	8.6	10.6
American white	10.3	11.4	9.2	11.6	8 9	11.0
Melanesian	10.9	12.3	10.0	12.3	9.3	11 7
	Lower					
	L	B	L	B	L	B
Australian aborigine	12.3	11.9	12.5	11.7	11.9	11.1
Bantu	11.0	10 5	11.0	10 3	11 1	10 4
Bushman	10 9	10 2	10 6	10.1	9.9	9.6
American white	11 2	10.3	10 7	10 1	10.7	9 8
American white	11 4	10.3	10.8	10.0	10.9	9 8
Melanesian	9.7	11.3	11 1	10 7	11 3	10 6

* Reproduced from Stein and Epstein (1934) by permission of the *Journal of the American Dental Association*
† Samples for each tooth range from 66 to 499

TABLE 2
LENGTH (L) AND BREADTH (B) OF THE MOLAR TEETH OF MELANESIANS IN MILLIMETERS*
Sample Sizes Range from 120 to 153 Teeth

	Upper					
	M1		M2		M3	
	L	B	L	B	L	B
M	11 1	12.5	10 1	12 5	9 2	11 9
F	10 8	12.1	10 0	12.0	9 5	11 4
	Lower					
M	11.3	11 4	11 2	10.8	11 5	10 7
F	11 6	11 1	11.0	10.5	11 1	10 4

* Reproduced from Stein (1936) by permission of the *Encyclopædia Sexualis*

even in the western Pacific, are incompatible with Heyerdahl's claim of an American origin of the Polynesians."

One of the most frequently noted and discussed dental traits is that of Carabelli's cusp of the maxillary M1 (also called fifth cusp, fifth lobe, mesiolingual elevation, or Carabelli's tubercle). Classically, it is an elevation of the enamel on the lingual side of the mesiolingual cusp, about halfway between its summit and the neck of the tooth. Its size and the groove that delineates it are extremely variable. According to Shapiro (1949), it occurs most often on the upper M1, but may be found, in about the same relative position, on all of the deciduous and permanent molars.

In his Table 21 Pedersen (1949) gives a useful racial summary of this trait complex (TABLE 5).

TABLE 5

PERCENTAGE INCIDENCE OF UPPER MOLARS WITH CARABELLI'S CUSP AND PIT IN VARIOUS RACIAL AND ETHNIC GROUPS*

Group	Author	Percentage with Carabelli's cusp		
		M3	M2	M1
Russians (20)	Batujeff	—	—	10.3 (53.8)
Swiss (313)	M. de Terra	1.35 (0 85)	0 22 (0 85)	11 2 (17 6)
Dutch (38)	Bolk	—	21 7‡	17.4 (44.3)
Hungarians (189)	v. Lenhossek	—	0 0 (0 0)	37.5‡
Germans (81)	Fabian	2 69 (4 71)	1.06 (7.17)	19.24 (51 07)
Finns (129)	Hjelmman	3.9 (5 4)	0.7 (3.7)	12 9 (9 2)
Lapps (159)	Kajava	0.0 (1.2)	0 0 (0.7)	3.4 (8 0)
Pecos Pueblo Indians (225)	Nelson	—	—	8.8§
New Pomeranians (151)	Janzer	6–12 times on 436 teeth	1.3	22.9

* Reproduced from Pederson (1949) by permission of *Meddeleser om Grønland*
† The figures in parentheses are additional percentages with Carabelli's pit.
‡ Cusp plus pit.
§ No pit mentioned

In a study of 200 molars of 9-year-old Iowa children, Meredith and Hixon (1954) found the Carabelli tubercle to be moderate to large in 60 per cent and absent in 16 per cent. In 70 per cent there was an observable side difference, right and left

The most thorough genetic analysis is that of Kraus (1951). From 8 pedigrees he concludes that "a genetic interpretation based upon the assumption of two allelic autosomal genes without dominance, or, in other words, with 'intermediate' dominance in heterozygotes" He postulates 3 genotypes, corresponding to 3 phenotypes: *cc* with complete absence of the trait, *CC* with full expression, and *Cc* with variable and less expression. FIGURE 3 (Kraus, 1951), illustrates his hypothesis.

Another accessory cusp often noted is the paramolar tubercle of Bolk, located on the buccal surface of both maxillary and mandibular teeth. However, Dahlberg (1945*a*) feels that the tubercle "should be called simply a parastyle or protostylid . . . and retain only the significance of a stylar cusp."

The congenital absence of upper I2 is said by Rushton (1953) to be a simple

mal" (nonshovel) tooth from the ancestral "abnormal" (shovel) tooth. In a study that accepts the fact that the trait, plus lateral ridges and grooves and rounding, is a stock-linked complex (Mongoloid), Riesenfeld (1956) concludes as follows: "A previously assumed Mongoloid cline from Indonesia, through Micronesia, to Polynesia, is confirmed by a west-to-east cline in the frequency of shovel-shaped incisors and rounded laterals. Such a cline and the fact that shovel-shaped incisors are much more frequent among American Indians than



FIGURE 2 Lower molars, showing cusp numbers and patterns Reproduced from Dahlberg (1945*a*) by permission of *Journal of the American Dental Association*

TABLE 4
FREQUENCY OF SHOVEL-SHAPED MAXILLARY CENTRAL AND LATERAL INCISORS*

	Median incisors					Lateral incisors				
	No of teeth	Shovel-shaped	Semi-shovel-shaped	Trace	None shovel-shaped	No of teeth	Shovel-shaped	Semi-shovel-shaped	Trace	None shovel-shaped
Chinese female	208	82 7	12 5	1 0	3 8	208	68 8	13 5	1 0	3 4
Chinese male	1094	66 2	23 4	1 8	7 8	1094	56 9	24 0	1 5	9 5
Japanese male	344	77 9	18 0	—	4 0	344	72 7	20 3	—	4 0
Indian	277	67 0	24 0	9 0	2 0	300	76 0	17 0	6 0	1 0
Mongolian	24	62 5	29 0	8 5	—	24	75 0	28 0	—	—
Hawaiian male	42	42 9	38 1	4 8	14 3	42	57 1	14 3	4 8	14 3
Hawaiian female	76	36 8	44 7	7 8	10 5	76	39 5	38 2	6 6	2 6
Eskimo	40	37 5	47 5	15 0	—	37	57 0	43 0	—	—
Melanesian	6	33 0	33 0	—	33 0	6	6 7	—	33 0	—
American Negro, male	618	4 9	7 6	33 0	54 5	618	4 5	12 8	38 0	42 1
American Negro, female	1000	3 6	8 0	32 6	56 0	1000	3 8	11 1	35 1	47 5
White male	1000	1 4	7 6	24 5	66 5	1000	1 4	8 8	36.4	50 0
White female	1000	2 6	5 2	21 8	70 4	1000	1 0	7 4	29 9	59 6

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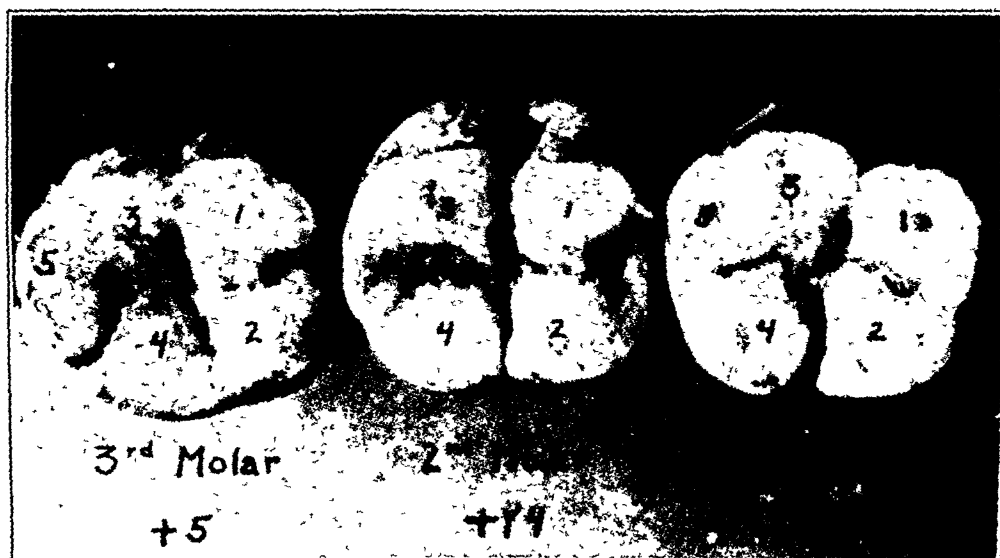


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Japanese male	344	77.9	18.0	—	4.0	344	72.7	20.3	—	4.0
Indian	277	67.0	24.0	9.0	2.0	300	76.0	17.0	6.0	1.0
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American Negro, male	618	4.9	7.6	33.0	54.5	618	4.5	12.8	38.0	42.1
American Negro, female	1000	3.6	8.0	32.6	56.0	1000	3.8	11.1	35.1	47.5
White male	1000	1.4	7.6	24.5	66.5	1000	1.4	8.8	36.1	50.0
White female	1000	2.6	5.2	21.8	70.4	1000	1.0	7.4	29.9	59.6

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even in the western Pacific, are incompatible with Heyerdahl's claim of an American origin of the Polynesians."

One of the most frequently noted and discussed dental traits is that of Carabelli's cusp of the maxillary M1 (also called fifth cusp, fifth lobe, mesiolingual elevation, or Carabelli's tubercle). Classically, it is an elevation of the enamel on the lingual side of the mesiolingual cusp, about halfway between its summit and the neck of the tooth. Its size and the groove that delineates it are extremely variable. According to Shapiro (1949), it occurs most often on the upper M1, but may be found, in about the same relative position, on all of the deciduous and permanent molars.

In his Table 21 Pedersen (1949) gives a useful racial summary of this trait complex (TABLE 5).

TABLE 5

PERCENTAGE INCIDENCE OF UPPER MOLARS WITH CARABELLI'S CUSP AND PIT IN VARIOUS RACIAL AND ETHNIC GROUPS*

Group	Author	Percentage with Carabelli's cusp		
		M3	M2	M1
Russians (20)	Batujeff	—	—	10.3 (53.8)
Swiss (313)	M. de Terra	1.35 (0.85)	0.22 (0 85)	11.2 (17.6)
Dutch (38)	Bolk	—	21 7‡	17.4 (44.3)
Hungarians (189)	v. Lenhossek	—	0 0 (0 0)	37.5‡
Germans (81)	Fabian	2 69 (4.71)	1.06 (7.17)	19 24 (51.07)
Finns (129)	Hjelmman	3.9 (5 4)	0 7 (3 7)	12.9 (9.2)
Lapps (159) .	Kajava	0 0 (1.2)	0 0 (0.7)	3.4 (8.0)
Pecos Pueblo Indians (225)	Nelson	—	—	8.8§
New Pomeranians (151)	Janzer	6–12 times on 436 teeth	1.3	22 9

* Reproduced from Pederson (1949) by permission of *Meddeleser om Grønland*
† The figures in parentheses are additional percentages with Carabelli's pit.
‡ Cusp plus pit.
§ No pit mentioned.

In a study of 200 molars of 9-year-old Iowa children, Meredith and Hixon (1954) found the Carabelli tubercle to be moderate to large in 60 per cent and absent in 16 per cent. In 70 per cent there was an observable side difference, right and left.

The most thorough genetic analysis is that of Kraus (1951). From 8 pedigrees he concludes that "a genetic interpretation based upon the assumption of two allelic autosomal genes without dominance, or, in other words, with 'intermediate' dominance in heterozygotes." He postulates 3 genotypes, corresponding to 3 phenotypes: *cc* with complete absence of the trait, *CC* with full expression, and *Cc* with variable and less expression. FIGURE 3 (Kraus, 1951), illustrates his hypothesis.

Another accessory cusp often noted is the paramolar tubercle of Bolk, located on the buccal surface of both maxillary and mandibular teeth. However, Dahlberg (1945*a*) feels that the tubercle "should be called simply a parastyle or protostylid . . . and retain only the significance of a stylar cusp."

The congenital absence of upper I2 is said by Rushton (1953) to be a simple

dominant with no sex limitation. Tobias (1955) notes also the frequency of missing lower I1 and calls attention to the fact that both of these frequently missing teeth are at contiguous bony intersections: upper I2 at maxillopremaxillary suture and lower I1 at mandibular symphysis. Thomsen (1952), on the basis of studies at Tristan da Cunha, suggests that missing incisors may be inherited differently, apparently with associated factors, as dominant, as recessive, and as sex-linked. Rantanen (1949) found missing and/or peg-shaped upper I2 in 2 per cent of 2218 Finnish young adults of both sexes; 9 different patterns of variability are given, but no sex differences were found. Schultz (1932, 1934) concludes merely that there is an "hereditary tendency" to eliminate the upper I2, with "different genetic modes in different families."

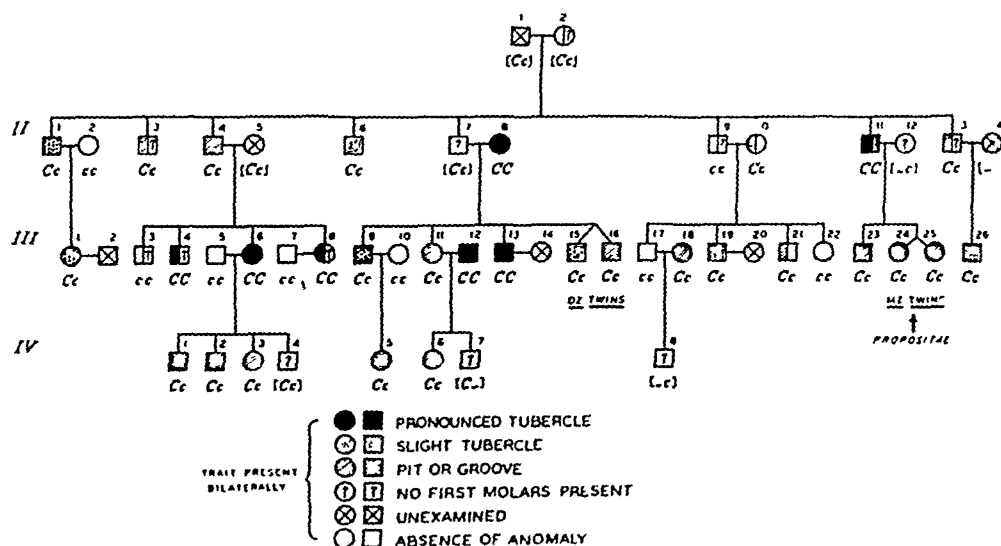
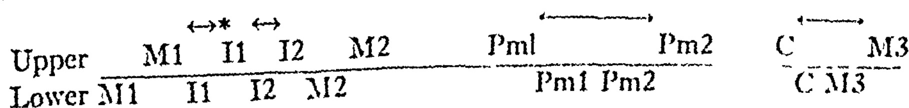


FIGURE 3 Pedigree of a Mexican family (Aros), showing Carabelli's anomaly, with proposed genotypes (*CC*, *Cc*, and *cc*). Genotypes that have been inferred from the phenotypes of relatives are enclosed in square brackets. Half-filled symbols indicate unilateral conditions Reproduced from Kraus (1951) by permission of *Journal of Human Genetics*.

The third molar is often congenitally missing. The best available data here are given by Hellman (1936), as summarized in TABLE 6 (see also Dahlberg, 1945).

Supernumerary teeth are often stated to be frequent in order of Negroid, Mongoloid, and Caucasoid. Tobias (1955) says the trait is dominant, while Rushton (1953) says it is probably dominant. The latter author notes that extra teeth are often found associated with cleidocranial dyostosis.

The eruption pattern of the teeth has received much attention Schultz (1935) has done the basic research in primate patterns, including man (see also Krogman, (1930). Specifically, Clements and Zuckerman (1953) present good schematic analyses for 188 chimpanzees and 166 gorillas with erupting permanent teeth:



* The symbol ↔ shows a possible variation in sequence in each of the 3 schemata.

For 2792 English children they offer the following (*see also* Clements *et al.*, 1953):

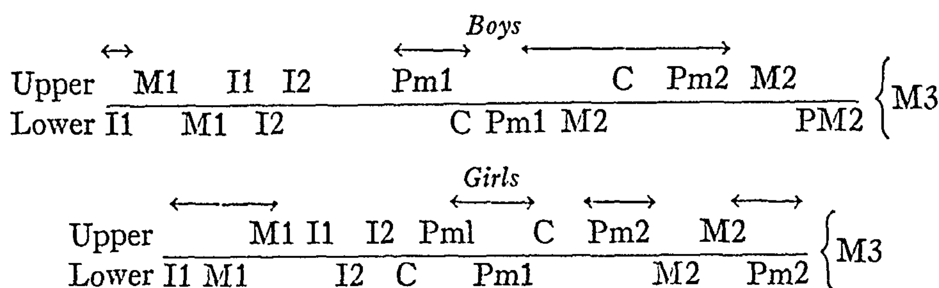


TABLE 6
THE RACIAL INCIDENCE OF MISSING MANDIBULAR THIRD MOLARS*

Group	Author	Missing
		(%)
Chinese† (19)		32.0
Eskimo (30)	Hellman	28.0
Eskimo	Goldstein	
males (363)		25.3
females (380)		28.9
European (61)	Hellman	20.0
European‡ (2112)	Golbirsch	10.3
Hawaiians	Chapple	
males (80)		17.5
females (58)		13.8
Mongoloid Buriats (21)	Hellman	17.0
American Indian (55)	Hellman	13 0
Australian Aborigine (20)	Hellman	13.0
American Negro (119)	Hellman	11 0
Early Egyptian§ (156)	Ruffer	3.2
West African Negro (49)	Hellman	0.0

* Reproduced from Hellman (1936) by permission of *Dental Cosmos*
Numbers in parentheses = sample size.
† Hellman's data are for males only.
‡ Combined males + females.
§ Sex not stated.

The problem of eruption sequence in early *Homo*, as contrasted with modern man, has been considered in detail by Garn *et al.* (1957, 1958). It is often said that in early man the teeth erupted in order of M1, I1, I2, M2, (P1, P2), C, M3, or M1, I1, M2, (P, P), C, and M3, while in modern man the order is (M1, I1), I2, (P1, C, P2), M2, M3, or (I1, M1), I2, (P, C, P), M2, and M3. In a study of modern American white children the authors differentiate between alveolar eruption, in which the M2, P2 sequence was observed with a frequency of 38 per cent, and clinical eruption in which P2, M2 is the rule. These investigators conclude that "on the basis of these findings, it is questionable whether the M2, P2 eruption sequence was truly more characteristic of fossil children than of contemporary juveniles."

For four population samples Steggerda and Hill (1942) offer useful summaries as shown in TABLE 7. Their data are graphically summarized in FIGURES 4 and 5.

On the genetic side it is generally accepted that eruption patterns (sequence, and especially timing) run in families (Tobias, 1955). Extreme acceleration or extreme retardation are not only familial, but are often associated with other

TABLE 7
ERUPTION TIME OF TEETH IN 4 RACIAL GROUPS*

Race		I1	I2	C	Pm1	Pm2	M1	M2	I1	I2	C	Pm1	Pm2	M1	M2
White	Male	7.9	9.2	11.8	10.7	11.5	7.0	13.1	6.9	8.5	10.9	11.2	11.9	7.0	12.4
	Female	7.6	8.8	11.4	10.5	11.3	7.0	12.6	6.6	8.0	10.2	10.6	11.6	6.6	11.9
Negro	Male	7.8	8.5	11.7	10.8	11.9	6.8	12.6	7.0	7.9	11.0	10.9	11.5	7.0	12.3
	Female	7.1	8.3	10.4	10.1	11.0	6.9	11.9	6.3	7.2	9.7	10.2	10.8	6.3	11.4
Maya	Male	8.4	9.3	11.8	10.3	11.6	6.9	12.5	7.4	8.4	11.2	11.1	12.0	6.8	11.9
	Female	8.3	8.6	10.9	10.0	10.9	6.7	12.1	7.2	8.1	10.3	10.2	11.2	6.7	11.5
Navajo	Male	7.7	8.8	11.1	10.1	10.9	6.7	11.6	6.8	7.7	10.3	10.2	11.2	6.3	11.6
	Female	7.7	8.7	10.6	9.9	11.4	6.6	11.4	6.5	7.4	9.8	10.2	11.0	6.4	11.2

* Reproduced with permission from Steggerda and Hill (1942) by permission of the *American Journal of Orthodontics and Oral Surgery*.

Because of insufficient numbers of second examinations, all second examinations and first examinations of the sixth and seventh years are included in the data on the central incisors and first molars of members of the white race Upper and lower jaws are set apart at the double vertical lines

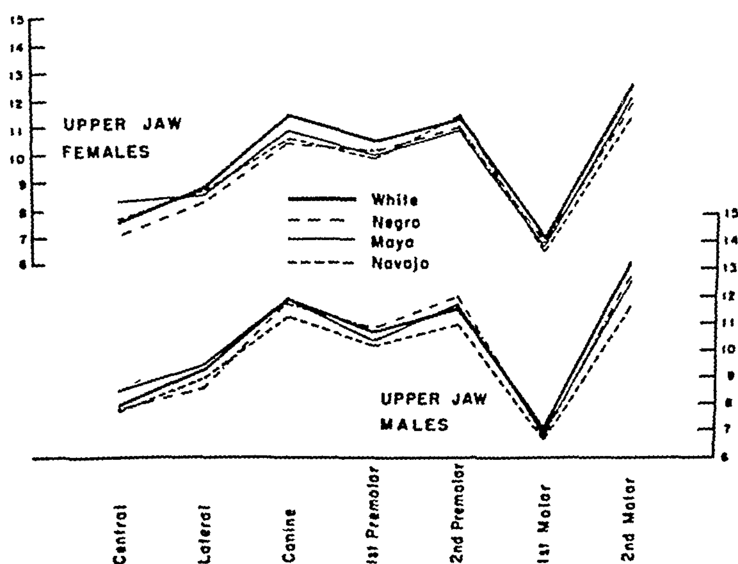


FIGURE 4 The variation in mean eruption time of the teeth of members of different races by age and sex. Reproduced from Steggerda and Hill (1942) by permission of *American Journal of Orthodontics and Oral Surgery*.

abnormalities (Rushton, 1953). Very early eruption is often associated with dominant polydactylism, while very late eruption is often associated with cleidocranial dyostosis. The retardation is progressive, that is, permanent teeth are more retarded than deciduous teeth; moreover, the roots are often deformed, and supernumerary teeth more frequent in this case.

The wear of the teeth is often noted in skull collections as a suggestion of age. As a rule, four stages are noted: (1) slight cusp abrasion; (2) moderate abrasion, with cusps worn level and dentine beginning to show; (3) medium abrasion, with dentine visible on most of the occlusal surface; and (4) marked abrasion, ranging from disappearance of the enamel from the occlusal surface to obliteration of the crown and invasion of the pulp cavity. Dietary habits play such an important role in wear that age can be only approximated.

An interesting anthropological observation in the dentition is that of ritual ablation (Hrdlicka, 1940). The practice is very ancient, going back to the Neolithic age in Europe and Asia and to pre-Columbian times in the Americas. The teeth knocked out were usually the upper incisors, but often canines and premolars were removed. Both sexes were involved, males more often than

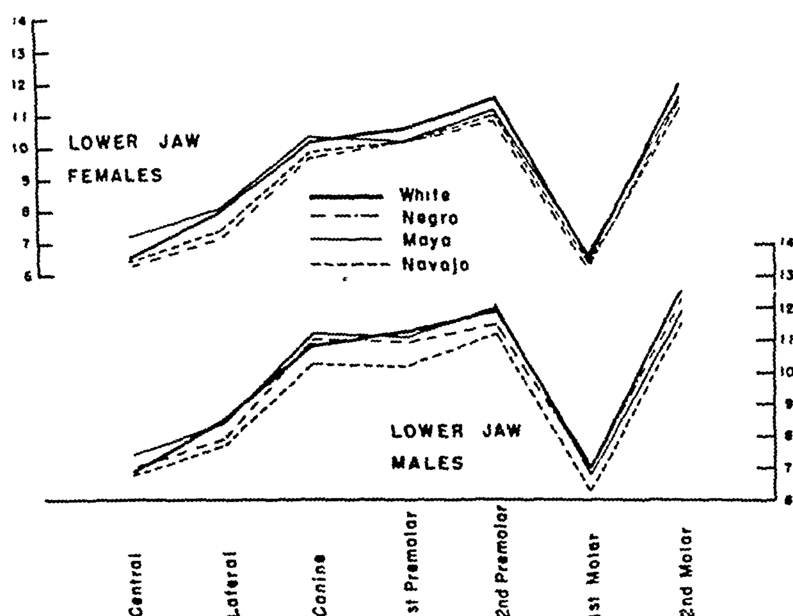


FIGURE 5. The variation in mean eruption time of the teeth of members of different races by age and sex. Reproduced from Steggerda and Hill (1942) by permission of *American Journal of Orthodontics and Oral Surgery*

females. The reasons given are usually those of propitiation or sacrifice, since the ablation was often done at puberty as a *rite de passage*, or that of the ability of males, especially, to endure pain.

The mandible must now command our attention in terms of the torus mandibularis, which is a bony outgrowth on the lingual side of the corpus. The best sources of information here are Drennan (1937), Grimm (1938), Hooton (1918), Schreiner (1935), and Weidenreich (1936). In a study of 100 skulls Drennan (1937) gives the anteroposterior (a-p) position of the torus as follows:

	I1	I2	C	P1	P2	M1	M2	M3
anterior limit	1	4	58	24	7	1	3	2
posterior limit	—	—	—	9	43	25	10	13

The racial incidence of the trait is shown in TABLE 8.

The most thorough studies of the heredity of dental traits are those of Lasker

(1950) and of Kraus (1956). Lasker principally reviews the congenital absence of teeth (M3, I2, Pm2, and I1), cusp patterns, Carabelli's cusp, shovel-shaped incisors, torus palatinus, and I1-I1 spacing. He points out the complexity of genetic analysis by using missing teeth as an example: in one family there may

TABLE 8
THE TORUS MANDIBULARIS* AND RACE

Group and No.	Frequency	
	Absolute	Per cent
Greenland Eskimo (215)	182	85
Smith Sound Eskimo (25)	18	72
Western Eskimo		
Male (350)	279	79.6
Female (360)	216	50.1
Children (62)	15	24.2
Iceland (56)	38	67.9
Ostyak		
Young (2)	1	50
Adult	3	50
Aged (6)	5	83 3
Lapp		
Male	—	26.8
Female	—	38.8
Children	—	12.9
Kistrand Lapp	—	53.3
Neiden Lapp	—	16.7
Ainu	—	24
Japanese		
Neolithic	—	62.1
Modern Kinai	—	14
Modern Kranto	—	9 4
Bushman		
Prehistoric (28)	14	50
Modern (50)	12	24
Scandinavian (164)	—	17
Norwegian		
Medieval male	—	24
Medieval female	—	10
Modern (M + F)	—	12
Chinese		
Prehistoric	—	25
Modern North Chinese	—	{15 = tubercle {40 = striation
Southern California Indian (46)	2	4 3
Italian (30)	1	3 3
Silesian (92)	41	44.6

* Unless otherwise specified all data are for the tubercle (mere rounded elevation) and striation (elongate elevation) types

be "peg laterals," in another the laterals may be absent, in another all lower I1 may also be absent, and in still another only one half of these teeth may be absent. He concludes that "the genes responsible in these four instances are apparently different . . . despite the fact that the anomalies seem to grade into one another." (References by Dahlberg, 1937, Gorry, 1955, Pedersen, 1949, and Keeler, 1935 are pertinent here.)

In TABLE 9 Kraus lists and categorizes a considerable number of traits, all of which have a genetic basis.

In TABLE 10 Kraus particularizes the odontological traits of a single tooth, that is, the lower Pm1.

TABLE 9

HEREDITARY ASPECTS OF HUMAN DENTITION*

Developmental traits
- Sequence of eruption, deciduous and permanent
- Age at eruption, deciduous and permanent
- Calcification of crown
- Time of molar-cusp coalescence

Mensurational traits
- Crown dimensions
- Number of cusps
- Relative size of molars
- Root length and number of roots

Morphologic traits
- Crown pattern: cuspules; wrinkles; ridges; grooves; pits; Carabelli's cusp; total shape
- Fused, bent roots
- Shovel-shaped incisors
- Enamel extensions and nodules
- Taurodontism

Anomalous traits
- Supernumerary teeth or roots
- Peg teeth, or congenitally missing teeth
- Paramolar tubercle of Bolk

Topical traits
- Diastemata, trema

Over-bite, over-jet, prodontism
- Shape of dental arches
- Incisor rotation

* Reproduced from Kraus (1956), by permission of the *American Journal of Orthodontics*

TABLE 10

INHERITED CHARACTERS OF THE MANDIBULAR FIRST PREMOLAR IN MAN*

(1) External lingual groove absent, 1 groove, 2 grooves
(2) Sagittal sulcus, mesiodistally between protoconid and metaconid, interrupted, uninterrupted.
(3) Deuteroconid a-p position mesial, distal, median
(4) Number of lingual cusps, each with independent apex from 1 to 5 cusps
(5) Mesial protoconid margin· absent, present
(6) Central protoconid ridge. bifurcated, nonbifurcated
(7) Accessory occlusal protoconid ridges none, 1 to 5 ridges
(8) Deuteroconid-protoconid relationship· joined deuteroconid, separate or independent deuteroconid

* Reproduced from Kraus (1956), by permission of the *American Journal of Orthodontics.*

In the area of dental pathology with a presumed genetic background we may note anodontia, frequently associated with anhidrotic ectodermal dysplasia. Rushton (1953) says that it is a sex-linked recessive. However, he cites Böök (1950) who says it is a simple autosomal dominant when it occurs with absent Pm1-2, prematurely white hair, and excessive sweating of palms and soles. When it is associated with absent I2 it behaves as a simple dominant with no

sex limitation. Hamano (cited by Rushton, 1953) says it is dominant when associated with missing premolars. Erwin and Corkern (1949) report missing M3 and Pm2 as a simple dominant with variable penetrance. McDonald (1949) says that anodontia in hereditary ectodermal dysplasia is incompletely recessive.

Hypocalcification of the enamel, whether due to deficient quantity, good quality, of enamel, or vice versa, is thought by Shear (1954) and Dreyer and Shear (1955) to be due to a dominant gene. Rushton (1953) says that enamel hypoplasia (agenesis of the enamel) is inherited as a dominant trait, and the enamel hypocalcification is dominant, with no sex differences. Tobias (1955) suggests that defective enamel may be transmitted either as a dominant or as sex-linked.

Dentinogenesis imperfecta, according to Hursey *et al.* (1956), who studied a "racial isolate" of 4000 to 5000 persons (Caucasoid, Negro, and American Indian), "appears to be transmitted as an autosomal dominant trait." These investigators go on to say that "wide variations in the manifestation of this trait were observed in patients heterozygous for the dominant gene."

Often associated with osteogenesis imperfecta or porphyria imperfecta is opalescent dentine with normal but incompletely calcified enamel. It is, says Rushton (1953), a dominant trait, Tobias (1955) concurs in this opinion.

The inheritance of cleft palate or cleft lip, or both, has received considerable attention. Fortuyn (1935) feels that cleft palate and lip "are a double recessive trait due to two genes, one of which is autosomal and the other one sex-linked This explains the fact that harelip is twice as common in the male sex as it is in the female " Test and Falls (1947) state that "the complex as a whole demonstrates the typical pattern of dominant Mendelian inheritance." The dominance is said to be "of irregular type," probably the result of variable expressivity

The problem of the total frequency of cleft lip and cleft palate has been summarized by Snodgrasse (1954) as shown in TABLE 11.

The most thorough study in this area is by Fogh-Andersen (1942) on a total of 708 Danish patients and Oldfield (1949) in 500 English children. A smaller, but very intensive, study is that of Snodgrasse (1954) on 55 white children studied at the Philadelphia Center for Research in Child Growth, Philadelphia, Pa. Fogh-Andersen concludes that cleft lip and cleft palate are genetically independent of isolated cleft palate; the first two are heritable anomalies. There is, he says, conditioned or incomplete dominance, with sex limitations to males and reduced penetrance. In most genetic milieus the responsible gene behaves as a recessive. The isolated cleft palate is rarely inherited as a simple dominant with sex-limitation to the female and reduced penetrance. Snodgrasse feels that the Philadelphia material does not support the hypothesis of simple dominant heredity. The data suggest, but do not prove, the possibility of simple recessive heredity with variable expressivity.

It must be pointed out that the cleft anomaly apparently may be due to other than genetic factors. Warkany (1947) uses the term phenocopy to denote the environmental simulation of an apparently genetic effect. Two sets of exogenous trauma may be at work: psychic (not generally adhered to) and mechanical. The latter is divided into extra-uterine and intra-uterine. Among the latter are amniotic bands, placenta previa, anoxia, finger in cleft,

tongue retained in embryonic position, deficient regional blood supply, chemical imbalance in the amniotic fluid, and fetal position. Among the former are maternal health (including infections, diabetes, and pelvic irradiation during pregnancy, age, nutritional status, and rank-order of child.

Faciodental Growth and Occlusion

We come now to a related but very important problem, namely, the possibility of inherited traits in the growth of the faciodental complex and in etiology

TABLE 11

TOTAL FREQUENCY OF CLEFT LIP AND CLEFT PALATE*

			Per 1000
1943 Grace	Pennsylvania	1:800 (250:202,501)	1.25
1950 Ivy	Pennsylvania	1:750	1 33
1947 Phair	Wisconsin	1:770	1.29
1949 Oldfield	England	1:600	1 66
1946 Hanhart	Zurich, Switzerland		0 80
1942 Fogh-Andersen	Denmark	1:665 (193:128306)	1 50
1924 Davis	Baltimore, Md.		
	(whites)	1:915 (17:15565)	1.09
	(negro)	1:1788 (7:12520)	0.55
1939 Edburg†	Sweden	1:960 (28:27000)	
1934 Grothkopp†	Germany	1:638 (74:47200)	
1929 Peron†	France	1:942 (106:100889)	

Frequency of Morphologic Types

	Phila Center		W B Davis		Danish Series (Fogh-Andersen)		British Series (Oldfield)	
Cleft lip	6	10.9%	133	14 2%	138	22 1%	88	17.6%
Cleft lip with cleft palate	28	50 9%	606	64 6%	360	57 6%	245	49.0%
Isolated cleft palate	21	38 1%	198	21.1%	127	20.3%	167	33 4%
Total	55	99 9%	937	99 9%	625	100 0%	500	100 0%

Sex Distribution of the Cleft Types

	Philadelphia Center						Danish				British			
	Males			Females			Males		Females		Males		Females	
	White		Negro	White		Negro	(Fogh-Andersen)				(Oldfield)			
	No	%	No %	No	%	No %	No	%	No	%	No	%	No	%
Cleft lip	6	100	——	—	—	——	90	65 2	48	34.8	53	60.2	35	39 8
Cleft lip with cleft palate	22	78 6	1	6	21 4	——	257	71 4	103	28.6	168	68.6	77	31.4
Isolated cleft palate	9	42 9	1	12	57 1	3	43	33 9	84	66 1	81	48 5	86	51.5
Total	37	67 3	2	18	32 7	3	390	62.4	235	37 6	302	60 4	198	39.6

* Reproduced from Snodgrasse (1954) by permission of the *Bulletin of the American Association for Cleft Palate Rehabilitation.*

† Maternity hospital records Figures for Sweden, Germany and France as cited by Fogh-Andersen (1942).

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"None of the angles studied bears a relationship to any other angle in the cranio-facial complex that is precise enough to be predictable. Furthermore, no definite relationship between any particular angle and any particular side of the polygon can be said to exist, and, finally, knowing that one particular side is relatively long or relatively short does not permit one to predict even roughly the length of any other side."

Kraus, *et al.* (1959) have also used X-ray films of the head and face. They agree with Wylie that "the utility of diameters and angles, which are in reality simply mental constructs, for recognizing the inheritance factor is brought into

TABLE 12
KEY TO BONE TRAITS*

Trait No	Description
	From lateral headfilms
1	External surface of occipital bone, from Bolton point to lambda, in MSP
2	External surface of calvarium, from lambda to bregma, in MSP
3	Profile of external surface of squamous portion of frontal bone, in MSP
4	External profile of supraorbital portion of frontal bone from nasion to a point above the supraorbital ridge, in MSP
5	Profile of nasal bones in MSP
6	Profile of cerebral face of orbital portion of frontal bone, in MSP
7	Profile of cerebral surface of cribriform plates of ethmoid bone in MSP
8	Profile of dorsum sella and planum sphenoideum in MSP
9	Profile of sella turcica in MSP
10	Profile of frontal process of zygomatic bone defined by orbital margin and anterior margin of temporal fossa, in MSP
11	Profile of anterior portion of maxillary alveolus and nasal surface of palatine process of maxillary bone, in MSP
12	Profile of oral surface of palatine process of maxillary bone, in MSP
13	Profile of inferior border of mandible, from gonion to menton, in MSP
14	Profile of posterior border of ramus of mandible, from apex of condyle to gonion, in MSP
15	Profile of the posterior margin of the mandibular symphysis, in MSP
16	Profile of the anterior margin of the mandibular symphysis, in MSP
	From frontal headfilms
17	Profile of mandible from a point on the right posterior border of the ramus intersected by the mastoid process to the equivalent point on the left side

* Reproduced from Kraus *et al* (1959) by permission of the *American Journal of Orthodontics*

serious question." Instead, these investigators turn to individual bones in p-a and lateral X-ray films "in terms of profile lines." Their Table XI (*see* TABLE 12) lists the 17 bone traits that they have used (*see also* FIGURE 6). Their study is based on 6 sets of triplets, 4 male, and 2 female, age range 9 years, 3 months, 11 days, to 15 years, 8 months, 18 days. The results of the analysis are given in TABLE 13.

These authors conclude, "We might speculate that the morphology of all the bones of the craniofacial complex are under the rather rigid control of hereditary forces." They add, however, that "it would seem that heredity governs morphology, but environment . . . has much to say how these bony elements shall combine to achieve . . . the harmonious (or unharmonious) head and face."

of occlusion. There can be no doubt of an over-all genetic control of species-limited organic growth. Cohen (1940) notes three planes or gradients of growth (cephalocaudal, dorsoventral, and mediolateral) and concludes that "the entire process is governed by genetic influences that determine growth of size of the body in all of its dimensions."

We may accept the foregoing in principle, but its application to the growth details of head, face, and teeth is another matter. To isolate a bone or a tooth, or even a tooth-bone complex, and to ascribe genetic specificity is to descend into doubt and uncertainty. However, we must take note of studies purporting to interpret the genetics of growth in the cephalic area.

No more detailed assertions of genetic specificity in face and teeth exceed those of Hughes and Moore (1941), Hughes (1942), Moore and Hughes (1942), and Moore (1944). For example, it is claimed that there is some sort of familial heredity in jaw displacements; inclination of the teeth; asymmetries (lateral displacement of chin and mandibular angles, level of orbital points, a-p malar position, palate and mandibular rotation, ramal height of mandible, length of mandibular corpus, and maxillary and mandibular dental heights); absolute size of mandibular ramus and corpus, palate, malar bone, palate height and width, and angle between the occlusal plane and the Frankfort Horizontal; mandibular angle; arch form; missing or extra teeth; depth of bite; cross-bite; and type of malocclusion. Statements such as these from Hughes and Moore (1941) are offered: "Probably 85 to 90% of the variability in both the deciduous and permanent dentitions can be ascribed to heredity"; or again, ". . . 70 to 80% of the size and configuration of the several parts of the mandible and the maxillofacial bones are hereditary." Hughes (1942) offers an escape clause from this overwhelming specificity when he says that "most of the craniofacial features, attribute as well as measurement, appear to be multiple factor traits. Single genes segregating normally seem to be the exception rather than the rule. Likewise, completely dominant genes and their recessive alleles are poorly represented" Rubbrecht (1939) tends to agree with Moore and Hughes (1942) when he observes that mandibular prognathism and maxillary retrognathism are inherited as irregular dominants, and that "the shape and size of the jaws are in great measure determined by heredity."

On sounder ground are the conclusions reached by Johnson (1949), based on C. R. Stockard's studies with dogs: (1) in size and form the cranium and the face are independent, (2) there is a lack of correlation between the upper face and the mandible, and (3) the size of the dental arch shows a far greater fluctuation than does the size of the teeth. In a sense, Korkhaus (1957) challenges the first conclusion. He states first that such pathological conditions as microcephaly, hydrocephaly, turrecephaly, chondrodystrophy, and dysostosis craniofacialis are all heritable patterns. Second, all involve a shortening of the anterior part of the cranial base, resulting in a reduced palatal length and consequently a retruded midface.

In recent years the problem of heredity in the craniofacial complex has been approached via serial lateral cephalofacial X-ray films in growing children An early study in this area is that of Wylie (1944), who employed certain planes and angles to establish so-called facial polygons, as seen (traced from the X-ray film) in normal lateralis. Wylie concluded as follows:

"None of the angles studied bears a relationship to any other angle in the cranio-facial complex that is precise enough to be predictable. Furthermore, no definite relationship between any particular angle and any particular side of the polygon can be said to exist, and, finally, knowing that one particular side is relatively long or relatively short does not permit one to predict even roughly the length of any other side."

Kraus, *et al.* (1959) have also used X-ray films of the head and face. They agree with Wylie that "the utility of diameters and angles, which are in reality simply mental constructs, for recognizing the inheritance factor is brought into

TABLE 12
KEY TO BONE TRAITS*

Trait No	Description
	From lateral headfilms
1	External surface of occipital bone, from Bolton point to lambda, in MSP
2	External surface of calvarium, from lambda to bregma, in MSP
3	Profile of external surface of squamous portion of frontal bone, in MSP
4	External profile of supraorbital portion of frontal bone from nasion to a point above the supraorbital ridge, in MSP
5	Profile of nasal bones in MSP
6	Profile of cerebral face of orbital portion of frontal bone, in MSP
7	Profile of cerebral surface of cribriform plates of ethmoid bone in MSP
8	Profile of dorsum sella and planum sphenoideum in MSP
9	Profile of sella turcica in MSP
10	Profile of frontal process of zygomatic bone defined by orbital margin and anterior margin of temporal fossa, in MSP
11	Profile of anterior portion of maxillary alveolus and nasal surface of palatine process of maxillary bone, in MSP
12	Profile of oral surface of palatine process of maxillary bone, in MSP
13	Profile of inferior border of mandible, from gonion to menton, in MSP
14	Profile of posterior border of ramus of mandible, from apex of condyle to gonion, in MSP
15	Profile of the posterior margin of the mandibular symphysis, in MSP
16	Profile of the anterior margin of the mandibular symphysis, in MSP
	From frontal headfilms
17	Profile of mandible from a point on the right posterior border of the ramus intersected by the mastoid process to the equivalent point on the left side

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serious question." Instead, these investigators turn to individual bones in p-a and lateral X-ray films "in terms of profile lines." Their Table XI (*see* TABLE 12) lists the 17 bone traits that they have used (*see also* FIGURE 6). Their study is based on 6 sets of triplets, 4 male, and 2 female, age range 9 years, 3 months, 11 days, to 15 years, 8 months, 18 days. The results of the analysis are given in TABLE 13.

These authors conclude, "We might speculate that the morphology of all the bones of the craniofacial complex are under the rather rigid control of hereditary forces." They add, however, that "it would seem that heredity governs morphology, but environment . . . has much to say how these bony elements shall combine to achieve . . . the harmonious (or unharmonious) head and face."

There is not much doubt that the foregoing study is proceeding in the right direction: the results speak for themselves. However, a word of caution is necessary, namely, that if proof of hereditary specificity be based on absolute trait identity, the limitations of the X-ray technique must be evaluated, because the roentgenographic cephalometric method does not permit of 100 per cent accuracy. A relative identity, not an absolute one, can be the only criterion.

A bit farther afield, before we turn to the occlusion, is the inheritance of

TABLE 13

CONCORDANCE (+) AND DISCORDANCE (−) IN INDIVIDUAL BONE TRAITS*

Trait No	Triplet set																	
	103			106			107			108			109			111		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	−	+	−	−	−	+	+	−	−	−	−	+	+	−	−	+	+	+
2	+	−	−	−	−	+	+	−	−	+	+	+	−	−	+	−	+	−
3	−	−	+	−	−	+	+	+	+	+	+	+	+	−	−	−	+	−
4	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	−	−	+
5	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	−	+	−
6	−	−	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+
7	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+
8	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−
9	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	+	−
10	−	−	+	−	−	+	+	−	−	−	−	+	+	+	+	−	−	+
11	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−
12	−	+	−	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−
13	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	+	−	−
14	−	+	−	+	+	+	+	−	−	+	+	+	+	+	+	−	−	+
15	−	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	−	−
16	−	+	−	+	+	+	+	−	−	−	−	+	+	+	+	−	+	−
17	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	+	−
Total number of concordances	1	4	4	7	7	15	17	3	3	4	5	13	16	14	15	5	9	6
Frequency of concordances	0.06	0.23	0 23	0 41	0 41	0 88	1 00	0 18	0 18	0 23	0 29	0 76	0 94	0 82	0 88	0 29	0 47	0 35
Zygosity indication		4			3			1			3			5			4	
Actual zygosity		4			3			1			3			5			4	

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certain facial features. In a general study of 7 generations of faces Lebow and Sawin (1941) noted long versus round faces, ear prominence, nose, and chin cleft. They concluded that long faces seemed to be dominant due to the "fortuitous recombination" of many genes. In a much more detailed report Pfannenstiel (1951) studied the faces of the Swiss of 3 Cantons (203 males, 208 females, aged 5 to 68 years). Conclusions were as follows. (1) height of the integumental upper lip, chin height, and mucous lip height are due to multiple genes, showing no dominance; (2) a thick, swollen integumental upper lip seems to be dominant; (3) the so-called Hapsburg lower lip is based on factors for a thick lower lip plus prodontia, which are independent: (4) the subintegu-

mental lower lip groove is a simple dominant; (5) the supramental (horizontal) chin groove and the intramental (vertical) chin cleft are simple autosomal dominants with almost 100 per cent penetrance but variable expressivity (the vertical cleft is less often seen and has lower penetrance).

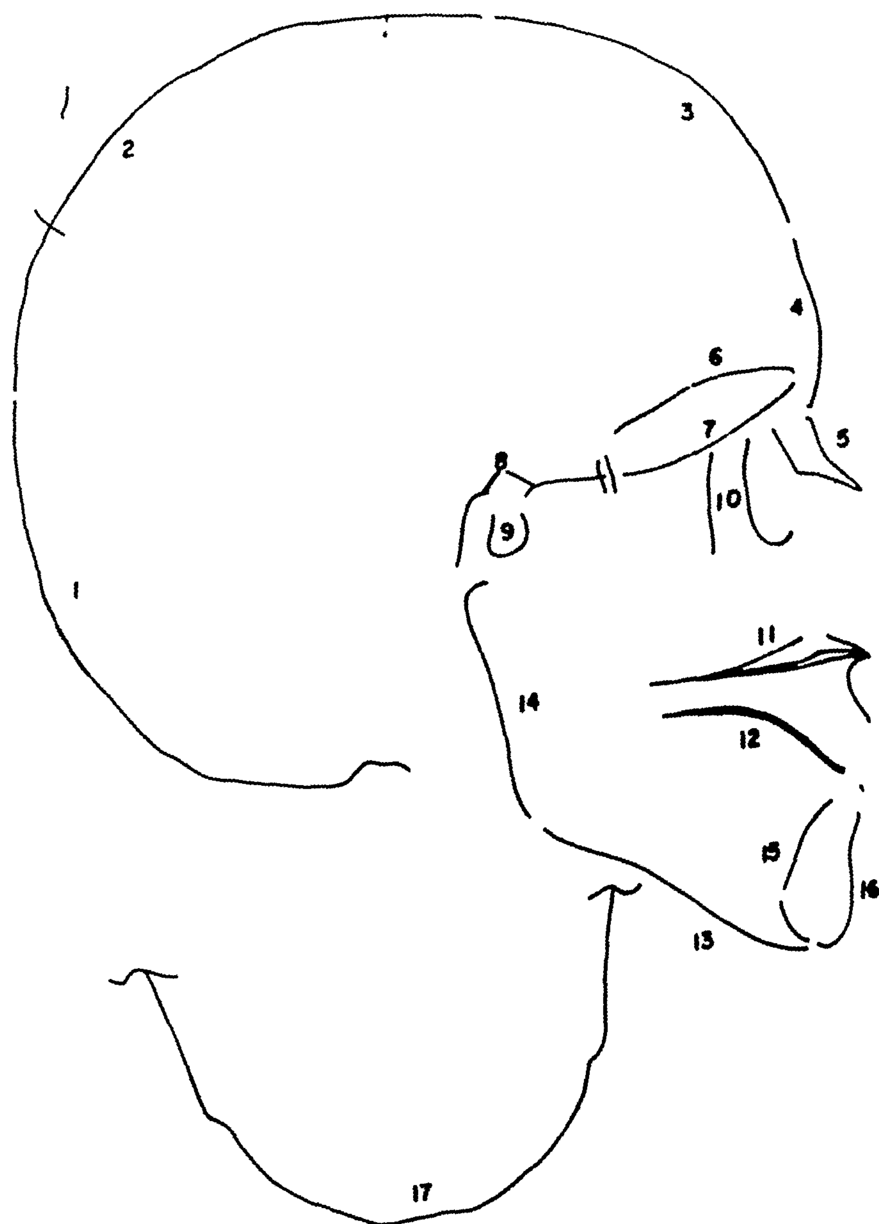


FIGURE 6 The identification of individual bone traits See TABLE 11 for key Reproduced from Kraus *et al.* (1959) by permission of *American Journal of Orthodontics.*

In the studies of Stockard on dogs, very extreme breeds were crossed, for example, the English bulldog with the dachshund, the former with marked cephalofacial achondroplasia, the latter with limb achondroplasia. It is no wonder, therefore, that Johnson (1949) concluded: "In the light of the evidence here presented on the relation of germinal constitution to skull form, there is room for little doubt that the primary factors of dental malocclusion in the dog are in the main an inherited condition."

On the genetic aspect of dental caries Rushton (1953) sums up quite fairly: "There is no satisfactory evidence that susceptibility to dental caries in Man is affected by genetic factors, although it is likely that that is the case."

Mention might be made here of the use of the teeth is forensic medicine for identification purposes. Scott (1954) discusses this problem in great detail.

Normal

Class I, Type 1

Class I, Type 2

Class I, Type 3

Class I, Type 4

Class I, Type 5

Class II, Division 1

Class II, Division 2

Class III, Type 1

Class III, Type 2

Class III, Type 3

Class IV

Cross-bite, anterior

Cross-bite, posterior

Open-bite, anterior

Open-bite, posterior

Unilateral

Missing teeth
(Arabic numbered-permanent)
(Roman numbered-deciduous)

Supernumerary teeth (numbered)

Photograph only (facial)

Unknown

Deciduous dentition

FIGURE 7 Symbolic representation of the observations in Asbell's study Reproduced from Asbell (1947) by permission of *American Journal of Orthodontics*.

TABLE 14

THE INCIDENCE OF DENTAL CARIES IN PREHISTORIC PEOPLES*

Population groups	Author	Percentage of total individuals	Percentage of total number of teeth
American Indian			
General	Mummery	—	9.5
Central America	Mummery	—	4.9
North America	Mummery	—	5.1
South America	Mummery	—	5.8
Pecos	Bodecker	—	3.0
Pecos	Rihan	47.9	—
Pecos	Nelson	47.0	—
Lenape	Hrdlicka	12.0 M	—
		16.0 F	—
California	Leigh	25.0 general 12–36, according to district	—
Kentucky	Leigh	30.0	—
Sioux	Leigh	12.0	—
Arikara	Leigh	28 0	—
Zuni	Leigh	75.0	—
Wisconsin	Fisher	21.7 M 33.8 F	—
Peruvian	Leigh	35.0	—
Canadian	Leechman	17.0	—
Eskimo			
	Mummery	—	1 4
	Leigh	—	1.0
	Goldstein	7.1 M 6.0 F	—
Egypt			
Badarian	Derry	5 4	—
Pyramid Age	Derry	17.6	—
XI–XII Dynasties	Derry	5.0	—
General	Leigh	12.0	—
Tepe Hissar, Iran			
Mediterranean	Krogman	43–71 M 43–64 F	—
Proto-Nordic	Krogman	55–57 M 43–77 F	—
Guam	Leigh	18 0	—
Sweden	Gunther	8 0	—
England and France			
Stone Age	Mummery	2 9	—
Bronze Age	Mummery	21 8	—
Iron Age	Mummery	40.0	—
Romano-British	Mummery	32 0	—
Anglo-Saxon	Mummery	15.0	—
French Neolithic	Mummery	1 5	3.0(?)
European mesolithic			
	Vallois	2 4–3 8	—
	Gunther	18 2–42 6	—

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Discussion

The oral structures, and beyond all doubt the dentition, are excellent materials for the study of both racial and genetic traits (in fact, raciogenetic might be a better term, or at least more useful one). The mammalian teeth form what may be called caenotelic or caenogenetic structures; that is, in form and in number they are, in the evolutionary view, relatively recent and, in terms of environmental lability, they are very adaptive. If they do not have a high mutation rate, they certainly have a high selective value. The human dentition is no exception to these generalizations. The exhaustive racial summary by Lasker (1950) and the detailed trait analyses, even of a single tooth, by Kraus (1956) affirm this.

There are two other observations to make, namely, that the dentition per se is not completely a genetic isolate. There is abundant evidence that the teeth

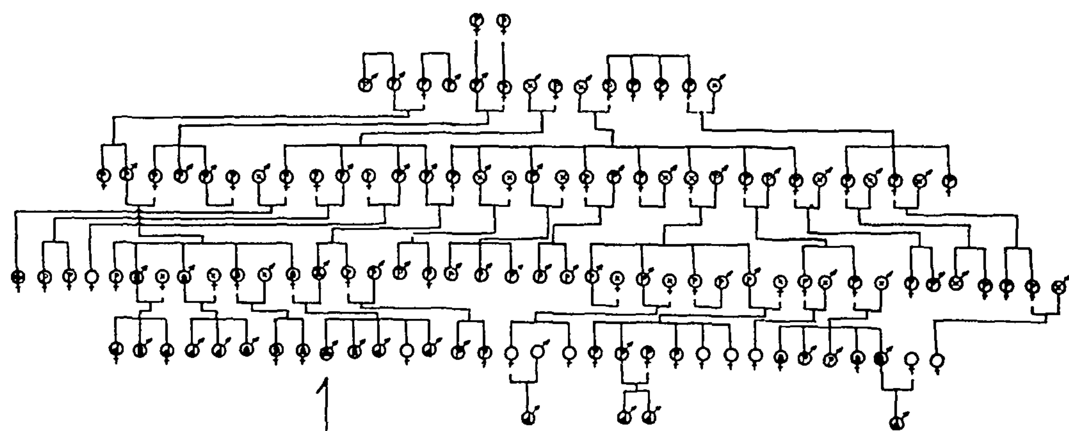


FIGURE 8 Pedigree of Patient A J (Cl II, Div 1, malocclusion—retrusive mandible) See FIGURE 7 for key to symbols Reproduced from Asbell (1947) by permission of *American Journal of Orthodontics*.

are often parts of a larger, more generalized, genetic complex. Furthermore, the dentition, within itself, shows regional genetic control or trait differences. The dentition, genetically speaking, is both interspecific (part of a trait complex) and intraspecific (variable within itself). This is to say that the dentition may be understood best in terms of the field concept (Dahlberg, 1945*a*).

In the entire dentition the incisors and the third molars betray the most variability, the premolars and M1-2 less, and the canines stand out as veritable Rocks of Gibraltar. The incisors show size, trait, and sex differences: a large upper I1 seems to be inherited; shovel-shaping is most frequent in upper I1 and I2, in order, male incisors, especially upper I1, are larger than female incisors; upper I2 is frequently congenitally absent, with lower I1 less frequently so. The molars also show size, trait, and sex differences. larger in Negroids (in order of M1, M2, and M3) and larger in males; racially different in cusp patterns (Y5, +5, and +Y4), especially in lower M1, variable in presence of Carabelli Cusp and the Paramolar Tubercle of Bolk. The canine preserves its phylogenetic and ontogenetic integrity. In the long mammalian history there has been but one canine tooth

$$\left(\frac{5\text{-}1\text{-}7\text{-}4}{4\text{-}1\text{-}7\text{-}4} \text{ to } \frac{4\text{-}1\text{-}4\text{-}3}{4\text{-}1\text{-}4\text{-}3} \text{ to } \frac{2\text{-}1\text{-}2\text{-}3}{2\text{-}1\text{-}2\text{-}3}\left(\text{to } \frac{1\text{-}1\text{-}1\text{-}2}{1\text{-}1\text{-}1\text{-}2}\right) ?\right);$$

it has always been at the corner (the German *Eckzahn* is a useful term), transitional between the transverse and sagittal moieties of the dental arch.

The dentition also shows its genetic linkage in that tooth size often varies in opalescent dentine and osteogenesis imperfecta, tooth eruption pattern may vary in cleidocranial dyostosis, and supernumerary teeth are often part of the

Table 15

The Incidence of Dental Caries in Primitive Peoples*

Population groups	Authors	Percentage of total individuals	Percentage of total number of teeth
Australian aborigines			
	Mattingly	18 0	1.6 (Mattingly, Nichols, Mummery, Campbell)
	Nichols	35.4	
	Mummery	20 5	
	Campbell	13.6	
	Campbell	3.7–18 2 according to district	0 7–1.6 according to district
(NE Austral.)	Campbell (L)	64 8	13 1
	Krogman	31 5 M	6.5 M
		35 0 F	6.3 F
Hawaiians			
	Chappel	—	1.5–45 9 M 6.7–51.5 F according to age, 40— to 60+
Eskimo			
	Collins (L)	27 8 M	—
		25 2 F	
	Friel (L)	53.8 M	2.4 M
		78.7 F	4.6 F
Bantu	Shaw	37 1	2.3
	Shaw (L)	—	6.5 M
			7.8 F
African			
	Proell (L)	24 8 uncivilized 66 2 civilized	—

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same condition. Certainly, eruption pattern as well as number of teeth are part of anodontia in varying degree.

The problem of cleft palate and cleft lip remains an open one. It seems that cleft palate with cleft lip may be inherited recessively, while cleft palate alone may well represent either genetic or environmental factors, or both.

It is in the realm of faciodental growth and the etiology of occlusion that important genetic advances are being made (mostly phenotypically, it is true, but genotypic data are beginning to appear). One thing seems to be established, namely, dimensions per se, or dimensions or planes forming triangles or polygons with angular relationships, will not yield much useful genetic growth patterning. A "single" dimension such as nasion-basion, or a "single" angle such as basion-sella-nasion, cuts across many different bones and, presumably, many different growth loci.

A new approach and a new technique are to be found in roentgenographic cephalometry. This involves the serial study of oriented X-ray films of head and face in p-a and lateral views, especially the latter. The use of individual bony traits as genetic markers holds hope for a better understanding of the hereditary factors in both faciodental growth and in occlusion (or malocclusion).

Oral structures, as parts of a larger cephalofacial complex, emerge dynamically as major contributors to knowledge in the related fields of anthropology and human genetics.

TABLE 16

THE INCIDENCE OF DENTAL CARIES IN EARLY HISTORIC AND PRESENT-DAY PEOPLES*

Population groups	Authors	Percentage of total individuals	Percentage of total number of teeth
Early historic			
Teutonic	von Lenhossek	36 5 M 36.4 F	5.8–11.7 according to date
	Schultka (500–1000 A D.)	67.3	10.9
English (18th century)	Krogman	65 8 M 88.2 F	19.7 M 31.3 F
Present-day			
American Indian			
Seminole	Krogman (L)		
Full-blood		91 7 M 89.8 F	29.7
Mixed-bloods		85 3 M 83 1 F	25.0
Chipewyan and Cree	Grant (L)	—	6 0 to 42 0 according to district
Canadian Indians	Price (L)	83 0–100.0 according to district	25 6–53.7 according to district
Navajo	Steggerda (L)	50 0 (age 30–35)	9.4
Maya	Steggerda (L)	72.0 (age 30–35)	8 9
Chinese	Anderson (L)	41.9 general 0 0–43 4 according to district	—
	Gunther (L)	40.0	—
Tibetans	Agnew (L)	37 2	—
Malay	Gunther (L)	7.0–11.0	—
Bavarians	Rose (L)	21 0 to 27.6 according to facial type	—

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THE TERATOGENIC EFFECTS OF HYPERVITAMINOSIS A UPON THE FACE AND MOUTH OF INBRED MICE*

Harold Kalter

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Introduction

The field of experimental mammalian teratology has caught the interest of individuals in a large number of disparate biological and medical disciplines, such as anatomy, pathology, genetics, radiology, embryology, pediatrics, gynecology, zoology, dentistry, preventive medicine, epidemiology, psychology, plastic surgery, pharmacology, nutrition, cardiology, endocrinology, and biochemistry. Workers in each discipline seek answers to their own questions, of course, but each will surely discover that much can be learned from the research carried out in other disciplines.

Studies in experimental mammalian teratology have become very numerous (Kalter and Warkany, 1959*a*), and the number of known teratogens is relatively large. It is likely, despite contrary opinion, that each teratogen or class of teratogens has characteristic repercussions on embryonic transformation and growth; hence the continuing search for new teratogens is a legitimate one, aiming at discovering means of interfering with the development of specific organs or systems of the embryo that hitherto have not been disturbed teratogenically, and studying the resulting disturbances to learn about modes of normal and of "spontaneous" abnormal development.

There exists a wealth of studies on gene-induced dental anomalies, especially those in mice (Gruneberg, 1952); however, few experimental studies have been made of prenatal environmental influences on teeth. These were recently summarized (Kalter and Warkany, 1959*a*, Kreshover, 1959). Cleft palate and cleft lip, however, are rather common effects of teratogenic procedures, and these induced malformations have been studied intensively from various points of view (Warkany *et al.*, 1943, Kalter, 1954 and 1957; Nelson *et al.*, 1955; Walker and Fraser, 1957; Fraser *et al.*, 1957).

The teratogenic method employed in the study to be discussed is maternal overdosage with vitamin A. This method was discovered by Cohlan (1953 and 1954), and its effectiveness has since been confirmed several times (for example, Giroud and Martinet, 1955; Deuschle *et al.*, 1959). In our laboratory this technique has recently been applied to inbred strains of mice and has been found to produce in them severe congenital malformation of many parts of the body (Kalter, 1959; Kalter and Warkany, 1959*b*).

Among these malformations was a syndrome of anomalies of the face and mouth that is of especial interest because it contains features not previously described in the literature dealing with teratogenic studies.

Materials and Methods

Young adult female mice of the A/Jax, DBA/1Jax, and C3H/Jax inbred strains were placed with males of their own strains, one pair to a cage, and

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observed each morning thereafter for a copulation plug. The presence of a plug was taken to indicate that ⅓ day had passed since conception. The impregnated female was then placed in a cage by herself and, at 8⅓ or 9⅓ days* after conception, was given one administration of 10,000 I.U. of vitamin A by mouth by means of a blunt-ended 19-gauge needle and tuberculin syringe. The vitamin, originally containing 300,000 I.U. vitamin A palmitate/cc. in sesame oil,† was diluted with sesame oil to 50,000 I.U./cc., and 0.2 cc. was administered. The animals were fed Purina Lab Chow and fresh tap water ad libitum throughout pregnancy. They were sacrificed 17⅓ days after conception, that is, approximately 2 days before term; resorption sites were counted and the fetuses were removed, examined macroscopically, and then fixed in Bouin's solution for serial sectioning or in 95 per cent alcohol in preparation for staining of the skeleton by the alizarin technique.

Results

To the time of writing no strain differences in type or frequency of induced congenital malformations have been detected; therefore the treated females and their offspring will be discussed without distinction as to strain. The effects of the vitamin on embryonic development were widespread (Kalter, 1959; Kalter and Warkany, 1959*b*). This report, however, deals only with malformations of the face and mouth, which were most severe and varied after treatment at 8⅓ and 9⅓ days after conception.

In 9 of the 23 females treated at 8⅓ days after conception, all (a total of 60) conceptuses were resorbed. The other 14 females produced 55 young and had 52 resorbed implantation sites. Eighteen females were treated at 9⅓ days after conception In 1 female only was the entire litter (8 conceptuses) resorbed. The other 17 females had 97 young and 34 resorbed implantation sites.

On external examination the 152 young possessed the following congenital malformations of the face and mouth, exclusive of those of eyes and ears (the number in parentheses being the per cent showing the defect). At or near the corners of the mouth there occurred dermal tabs and hillocks of varying length and size (62.5 per cent). In many of the animals without such dermal appendages there occurred microstomia, that is, reduction in size of the oral aperture, ranging from a moderate decrease to apparent obliteration of the oral opening (33.6 per cent). Accompanying microstomia and seeming to parallel it in severity was a variable reduction in size of the proximal end of the mandible. In addition to this mandibular defect, there occurred micrognathia, that is, shortening of the mandible distally, giving a retrognathic appearance (17 8 per cent). Shortening of the upper jaw appeared in a few animals (11.2 per cent). In this study median cleft mandible occurred rarely (2.0 per cent), but in subsequent series it has appeared more frequently (see FIGURE 1 for illustrations of above-discussed anomalies).

Ninety-three of the animals' mouths could be opened and the palate examined; 52 of these had cleft palate. The remaining 59 had microstomia, which prevented examination of the palate without damaging the mouth, or were too fragile for this examination. Six of the 59 had unilateral or bilateral cleft lip

* Females were also treated at 7⅓, 10⅓, 11⅓, or 12⅓ days after conception, but the results of treatment at these times are not included in this paper

† Obtained from Hoffmann-LaRoche Inc , Nutley, N J

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fore, of the total of 112 whose palates were examined in various ways, 70 had cleft palate (62.5 per cent). This, of course, is an underestimate, since most of the palates of the microstomic animals were not examined; in these animals, it was discovered, the palates are most likely to be cleft, since 18 of 19 cleared and sectioned microstomic specimens had this defect.

With the exception of cleft palate, these malformations occurred with roughly the same frequency as a result of treatment at 8⅓ days after conception and after treatment at 9⅓ days after conception. For dermal tabs these frequencies of occurrence were 54.5 and 67.0 per cent, for microstomia 36.4 and 32.0 per cent, for micrognathia 18.2 and 17.5 per cent, and for cleft palate 82.9 and 53.2 per cent, all respectively. The more frequent occurrence of cleft palate after the earlier treatment in pregnancy therefore must have been due

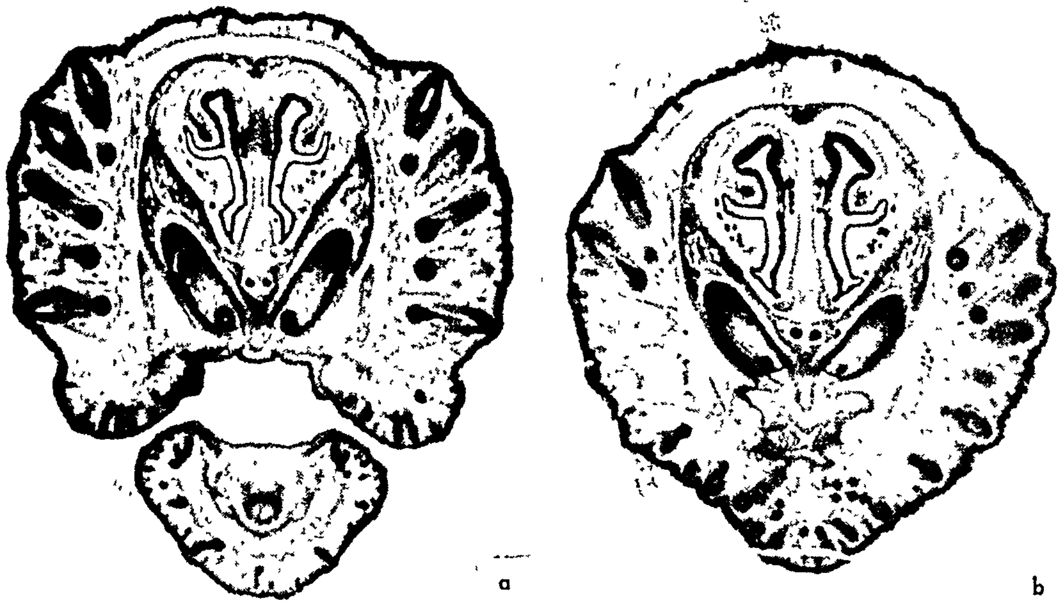


FIGURE 2 Frontal sections through the level of the upper incisors (*a*) Control; (*b*) experimental animal, showing minute mouth, thick oral epithelium, and short lower jaw.

to the fact that a greater proportion of nonmicrostomic animals were affected at that time than at the later time of treatment.

FIGURES 2 through 7 consist of comparable frontal sections from front to back through the heads of a control animal and an experimental animal from a female treated at 9⅓ days after conception; FIGURES 8, 9, and 11 are frontal sections through the head of an experimental animal from a female treated at 8⅓ days after conception. In such serially sectioned material, obtained from 9 animals treated at 8⅓ or 9⅓ days after conception, a remarkable array of defects was seen. The oral opening, as already mentioned, was variably reduced in size. Underlying this outward condition were extremely complex malformations of the mouth and its surroundings The mouth itself was often drastically reduced in size (FIGURES 2 and 3), sometimes to such an extent that the tongue was tightly encased and distorted (FIGURE 4). One of the causes of this was a reduction or obliteration of the lateral recesses of the mouth, as if

(all were A/Jax animals, a strain in which cleft lip with or without cleft palate occurs spontaneously in about 5 per cent of the young). Of the 53 without cleft lip, 16 were cleared for skeletal examination and 15 were found with cleft palate, 3 were serially sectioned and all were seen to have cleft palate. There-

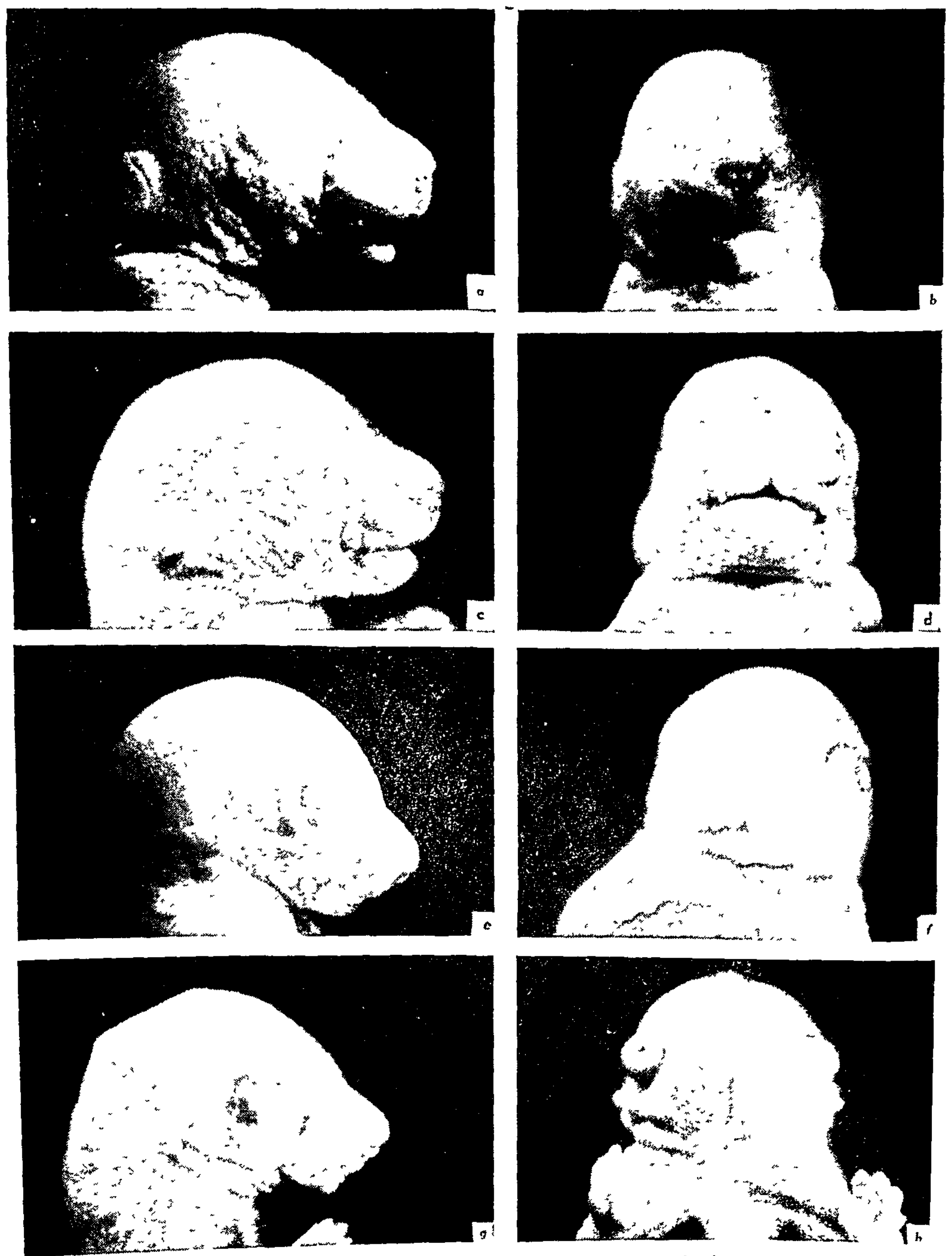


FIGURE 1. External appearance of the head (*a* and *b*) Control, (*c* and *d*) experimental animal, dermal tabs; (*e* through *h*) different grades of microstomia and mandibular maldevelopment.

At the fetal stage observed, the first lower molars in control animals are in the bell stage, the enamel organ is beginning the formation of the cusp pattern, and the connection between the enamel organ and the dental lamina is almost lost. Minute second molars are present. In general, anomalies of first molars consisted of their being absent, ectopic, misshapen, or smaller or larger than normal. Ectopia of the lower molars consisted in their being located in the tissue causing the obliteration of the lateral recesses of the oral cavity. Such molars were turned on their side with the cusp pattern vertical and facing mediad and with the dental lamina horizontal and joined to the oral epithelium

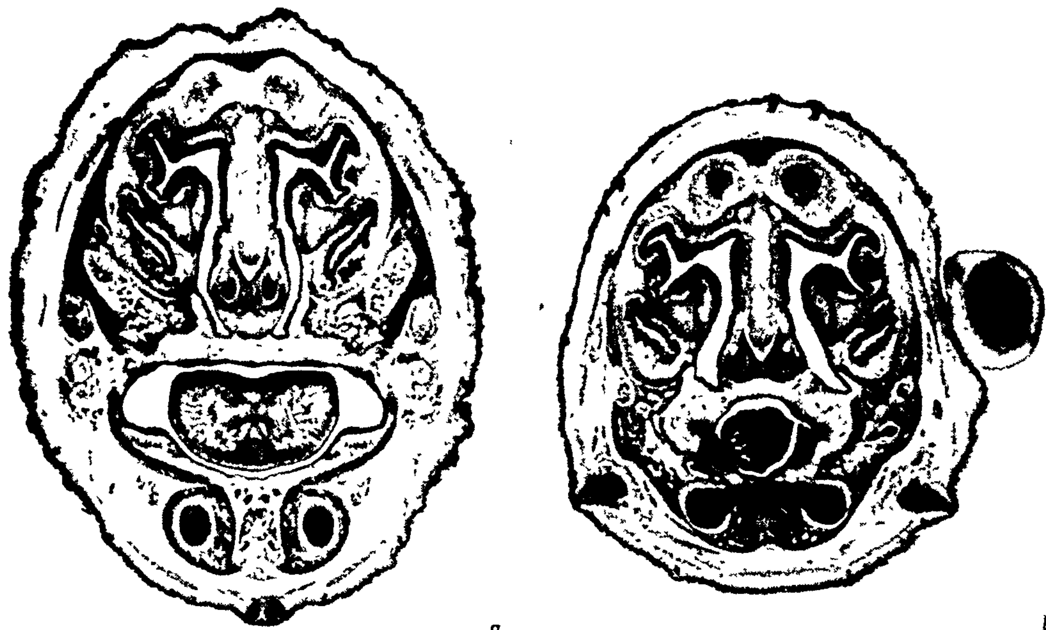


FIGURE 4 Frontal sections through preorbital region (*a*) Control; (*b*) experimental animal, showing massive bilateral maxillomandibular ankylosis, small infraorbital foramen, small mouth and small, compressed tongue, abnormal course of the common nasal meatus, displaced nasal cartilage, absence of masseteric musculature, absence of sublingual ducts, enlarged lower incisors, and other abnormalities

at the lateral edge of the oral cavity. Ectopic upper molars lay far lateral to their normal position, in a bizarre location in the cheek. In this case the dental lamina took an erratic course, but failed to connect with the mouth (FIGURE 9).

Ectopia of molars was invariably associated with the trapping, by pieces of heterotopic cartilage, of oral epithelium that was invaginated into buccal tissue. This will be more fully described below.

The second molars were either apparently normal, absent or, in the case of upper second molars, slightly shifted laterally. The last anomaly was probably entirely referable to the displacement of the gums caused by cleft palate. The upper first molars were also slightly shifted.

Various skeletal elements of the face were often markedly abnormal. The following description refers to the most severely affected specimens, usually the result of treatment at 9⅓ days after conception. As could be seen in

the recesses had been zippered up, leaving the abnormally thick oral epithelium projecting deeply into the tissue at the sides of the mouth (FIGURE 3). In addition, the oral cavity was diminished by the presence of the unascended and abnormally shaped palatine shelves, which fused in complex and varied ways with buccal surfaces of the mouth, leaving intricate and tangled oral pockets in the fused regions (FIGURES 4 and 5). Further back, the lateral and dorsolateral edges of the tongue became fused to gingival and palatine tissue, again leaving oral outpouchings between the lateral edges of the tongue and inner surfaces of the gums (FIGURE 6). As these outpouchings rapidly became occluded, all that was left of the mouth was a narrow, horizontal, crescent-

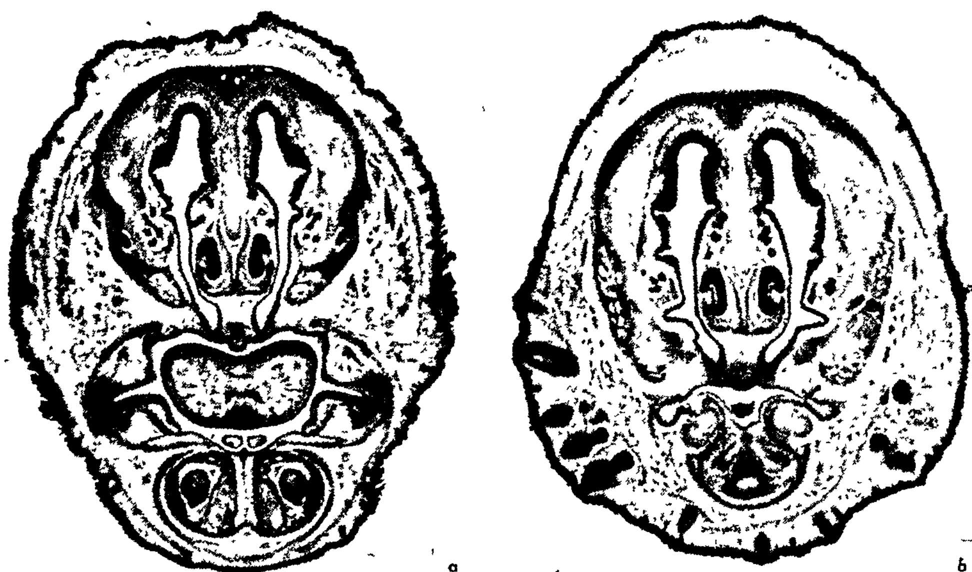


FIGURE 3 Frontal sections through the level of the internal nares (*a*) Control; (*b*) experimental animal, showing small mouth, heterotopic cartilage, invaginated oral epithelium and short mandible, and ventrally enlarged rostral portion of maxilla.

shaped space, identifiable as the nasopharynx, between the dorsal surface of the tongue and the basocranium (FIGURE 7).

Various anomalies were found in the dental structures themselves, for example, fused, supernumerary, absent, and ectopic teeth. Lower incisors were generally normal except for occasional enlargement or slight inward rotation. In an instance of fused upper incisors, it was found that the lateral borders of the "tooth" were so situated as to indicate that the incisors proper were more or less normally located; however, it was as though an intercalated wedge of dental tissue had become fused to them. The entire mass consisted, therefore, of a block of tooth much larger than the bulk of two incisors alone. A second incisive abnormality consisted of unilateral or bilateral supernumerary upper incisors. These were located posterior and lateroventral to the normally situated incisors and seemed as wide as, but shorter than, normal incisors (FIGURE 8).

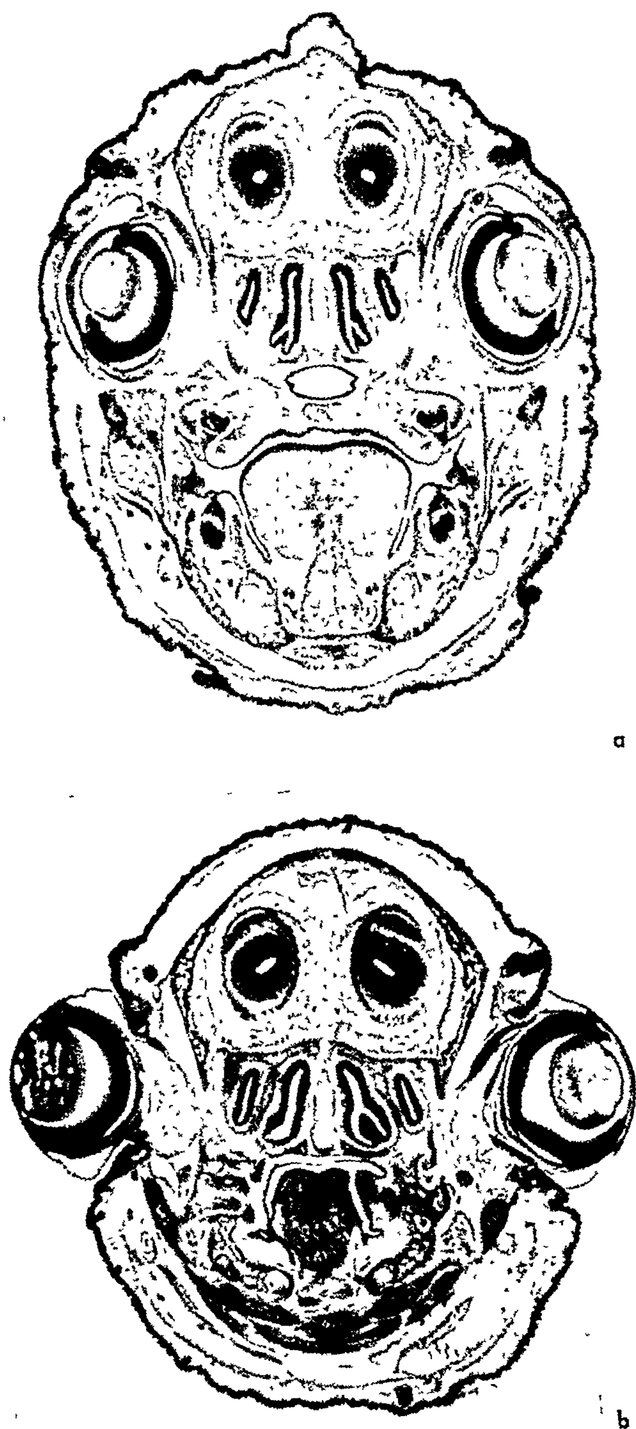


FIGURE 6 Frontal sections through the level of the first molars (*a*) Control; (*b*) experimental animal, showing absence of the first molars, the zygomatic process emerging from the region of maxillomandibular ankylosis (instead of from the crista facialis, as in FIGURE 5*a*), heterotopic cartilage large on the right and smaller on the left (lateral to the oral cavity), and beginning fusion of the tongue and gum on the right side. In this, as in several other sections of abnormal specimens, exophthalmia is present, being the product of absence of much of the skeletal supporting structure of the orbit Disorganized sublingual musculature is also seen

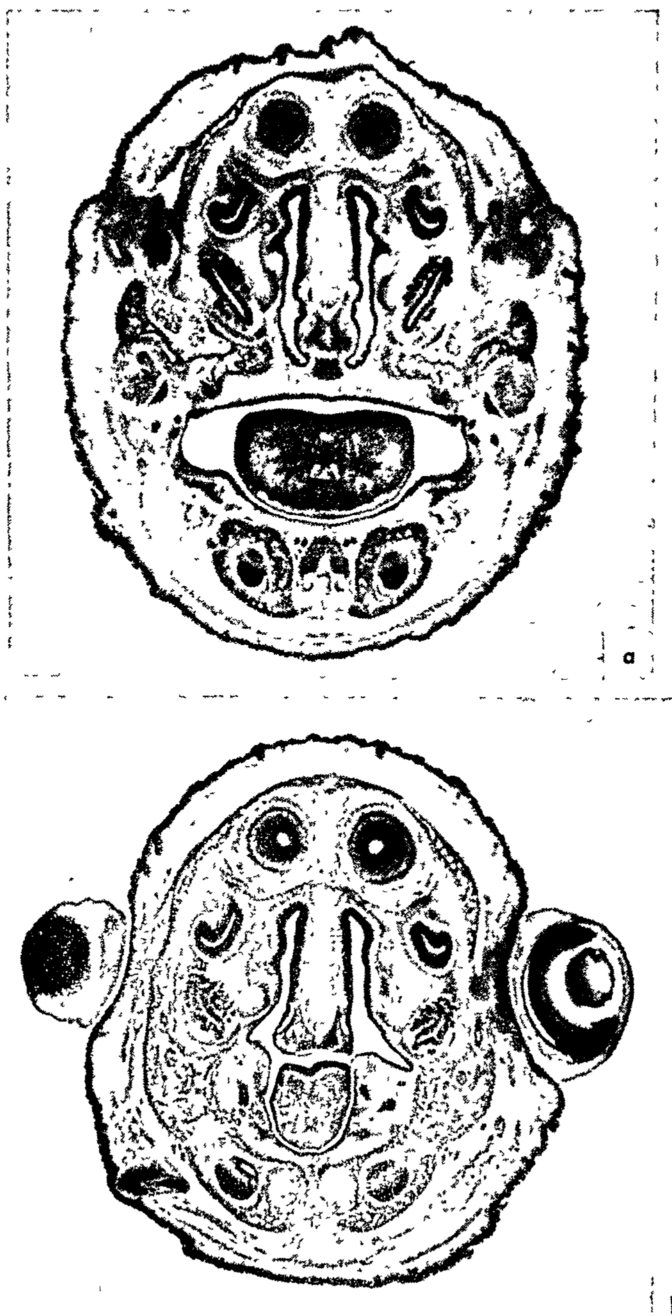


FIGURE 5 Frontal sections through the level of the anterior orbit (*a*) Control; (*b*) experimental animal, showing much the same variety of anomalies as in FIGURE 4*b* and also cleft palate, unascended palatine shelves fused to buccal tissues with cross sections of oral outpouchings in the regions of fusion, absence of parotid ducts, and heterotopic cartilage in the area of fusion of maxilla and mandible Contrast of the abnormal animal with its matching control shows also that the portion of the maxilla from which the zygomatic process normally originates is absent, with consequent absence of portions of skeletal elements of the orbit

rostral portion of the maxilla was seen to be situated too far laterally and to be enlarged and extended ventrally (FIGURE 3). This ventral enlargement increased rapidly, only slightly more posteriorly, extending ventromedially to form a thick and solid ankylosis with the dorsolateral margin of the mandibular body (FIGURE 4). The infraorbital foramen was very small. The ventrolateral arc of nasal cartilage was displaced laterally, and the base of the common nasal meatus projected far ventrolaterally instead of gently curving ventromedially

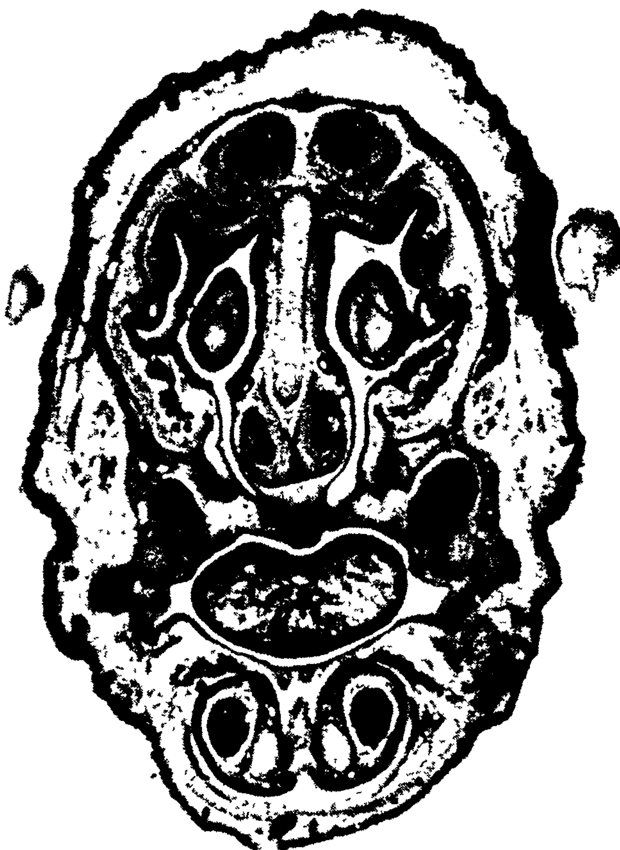


FIGURE 8 Frontal section of abnormal animal showing supernumerary upper incisors bordered by heterotopic cartilage ventrolaterally and by an abnormal spur of maxilla laterally. Compare with same section of control in FIGURE 3*a*

(FIGURE 4). Further posteriorly, the zygomatic process of the maxilla, which was larger than normal, originated far too ventrally from the area of massive ankylosis of maxilla and mandible (FIGURE 5). The crista facialis of the maxilla was absent, with the consequence that, instead of arching gracefully out and back, the zygomatic process emerged and coursed straight back, far too medially (FIGURE 6).

Proceeding posteriorly, the mandible gradually diminished and nearly disappeared, until only isolated osseous remnants around and close to the mouth were left. With the almost total disappearance of the mandible, Meckel's cartilage was also no longer present, and did not reappear. Several heterotopic,

cleared specimens (FIGURE 10) and as was confirmed in sectioned material (FIGURES 3 through 7), the mandibular ramus and its processes were entirely or nearly entirely absent, the mandibular body aberrant, and the maxilla and its various processes grossly deformed. At the level of the internal nares, the

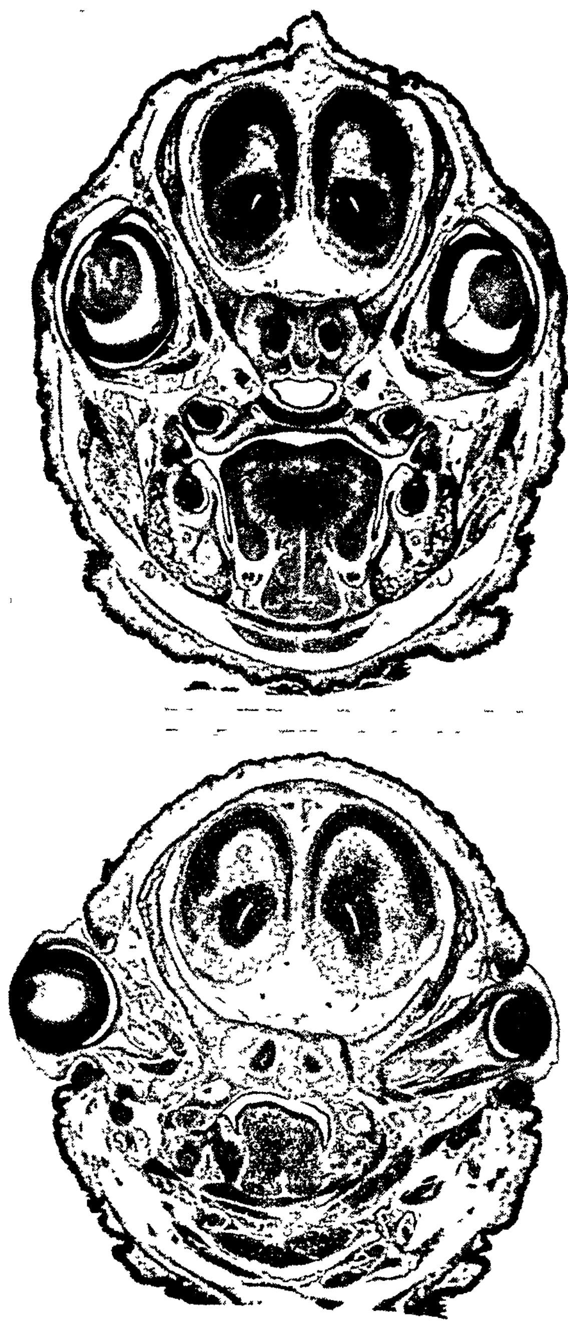


FIGURE 7 Frontal sections through the level of the optic nerve (*a*) Control, (*b*) experimental animal, showing almost complete absence of the mandibular ramus, heterotopic cartilage at the corners of the oral cavity, the tongue immobilized by fusion to gums, cleft palate, disorganized sublingual muscles, and other effects

variable-sized pieces of cartilage were present in areas surrounding the mouth, generally in or close to bone. Heterotopic cartilage appeared at the corners of the mouth near the lips (FIGURE 3), rapidly enlarging and, further back, fusing with heterotopic cartilage present in the zygomatic bone, to form large horizontal bars or other shapes of cartilage (FIGURE 11). Often the heterotopic cartilage engulfed the fingers of oral epithelium that projected laterally from the corners of the mouth, to form islands of this tissue. Proceeding posteriorly,

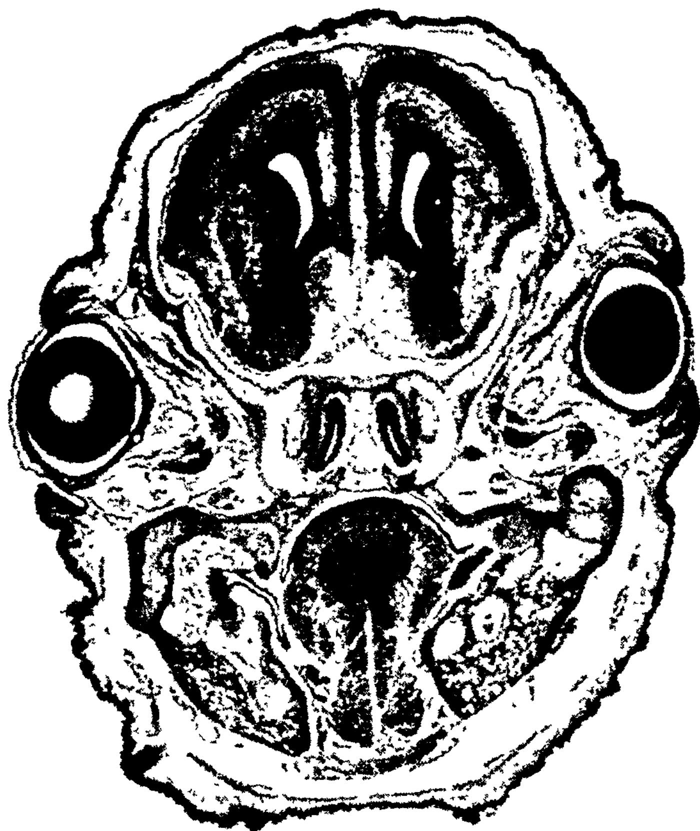


FIGURE 11 Frontal section of abnormal animal, showing fusion between heterotopic cartilage in zygomatic bone and that at corners of mouth Note also the bridge between the tongue and gum on the right and the dental anomalies

the zygomatic bone became greatly thickened as it joined the zygomatic process of the squamosal bone, investing and otherwise closely associating with masses of heterotopic cartilage as it did this.

Defects of various other soft tissues were also noted, among them disorganization and absence of sublingual and submaxillary muscles, absence of all parts of the masseter muscle, absence of the parotid ducts and of the sublingual glands and ducts, and unilateral absence of the submaxillary glands.

Discussion

Previous investigators using the method of hypervitaminosis A have found its teratogenic action widespread and powerful, and have largely concurred as

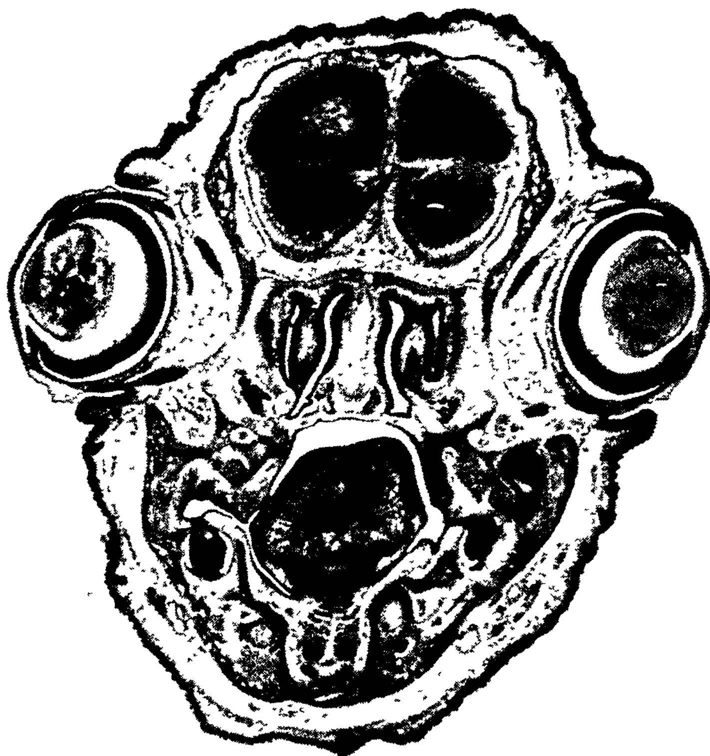


FIGURE 9. Frontal section of abnormal animal showing such effects as absent, ectopic, and abnormal molars Compare with same section of control in FIGURE 6a



FIGURE 10 Cleared skull of abnormal animal showing maxillomandibular ankylosis

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regards its effects. Certain discrepancies have occurred, however. For example, Cohlan (1954), treating rats, produced foreshortening of the maxilla and mandible, but found that the latter was usually "due to shortening of the corpus mandibulare, the ramus rarely being affected," whereas Deuschle *et al.* (1959), also using rats, found that "more proximal portions of the jaw" were affected. Furthermore, Giroud and Martinet (1958), using rabbits, reported a "complex case of microstomia," but apparently failed to affect the mouths of mice (Giroud and Martinet, 1959). Deuschle *et al.* (1959), however, produced a syndrome of dentofacial anomalies in rats greatly similar to the one described in the present paper for mice.

The animals described in this paper possessed a pattern of anomalies that presents certain features not often encountered in past teratogenic studies. Most induced anomalies of the face have consisted of fissures between various processes or decrease in size or absence of certain structures. In this study, on the other hand, were regularly found excesses of tissues, such as skin, bone, cartilage, and tooth.

It is probable that certain facets of this syndrome of dentofacial malformations were more immediate effects of the treatment and that others were more remote consequences of the earlier responses. To say which defects preceded and which followed, however, is not too easy in most cases, and a precise analysis must await pathogenetic investigations. It is not likely, however, that the dental defects were early results of the teratogenic agent; more probably they were mediated through and dependent upon anomalies induced in precursor and influencing tissues, such as ectoderm and mesoderm and, later, bone.

Studies such as this one, in fact, emphasize the interrelatedness and coordination of developmental events by showing how their deflection at crucial moments can reroute subsequent pathways and have unforseeable and far-reaching consequences Such studies, therefore, can be valuable in the elucidation of normal patterns of sequences of embryonic events.

Summary

This paper describes and discusses the congenital malformations of the face and mouth in the offspring of inbred female mice given a large dose of vitamin A about halfway through pregnancy.

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analogous to those targets influenced elsewhere in the body by the same agent; the influence of the age and developmental state of the individual tissue and the whole organism on the nature of the response, the variation in response from species to species; and, in some cases, such as that of vitamin D, the contributing influence of other dietary components such as calcium, phosphorus, and phytates on the response

The zeal, ability, and interest of the early investigators have served to introduce us into interesting areas judged to be of such importance to dental research that the planners of this monograph have chosen several other topics that touch upon some phase of the general theme assigned to me. In view of this fact and of the excellent recent reviews already mentioned, I have chosen to avoid repetition by limiting my discussion to three topics that involve certain interrelationships among nutritional and metabolic variables, host response, bacterial transmission, and susceptibility or resistance to oral disease.

Inheritance, Nutritional Influence, and Caries Susceptibility

The inheritance of anatomical characteristics of the teeth has been studied less thoroughly in man and in experimental animals than might be expected in view of the accessibility of the teeth in life and their durability in post-mortem material. An hereditary absence of the incisor teeth, a simple Mendelian recessive characteristic, has been reported by Greep.[20] This syndrome is not sex-linked in its inheritance and is characterized further by failure of the first molars to erupt and by partially erupted second and third molars.[21] This strain of rats is commonly known as incisorless (ia). Somewhat similar dental conditions were observed in the grey-lethal mouse[22-24] and in a lethal mutation in the rabbit.[25,26] Both syndromes were recessive and were associated with a lack or retardation of bone resorption

Tooth development in the ia rats was normal prior to the formation of the alveolar crypt. However, the bony crypt, which normally grows by resorption on its dental surface and by apposition on its outer surface, failed to provide adequate space for the growing tooth germs because of a lack of bone resorption. The epithelium of the enamel organ invaded the bone marrow spaces, and ectopic formation of dentin, enamel, and cementum led to ankylosis, failure to erupt, and the formation of odontomatoid structures[27] The administration of "parathormone" to the young ia rat resulted in the reduction of the incidence and severity of dental anomalies, pseudo-odontomes and, in most cases, eruption of the teeth when a sufficiently long period of parathormone therapy was provided.[28] The rats in which administration was started early and continued longest showed the most nearly normal development and eruption of the largest number of teeth. Shorter periods of treatment of the grey-lethal mouse with parathormone led to marked bone resorption without any improvement in the dental anomalies.[29]

Other grossly visible abnormalities such as a fusion of the first and second molars, supernumerary molars, and both simultaneous fused and supernumerary molars in the rice rat are under investigation to determine their pattern of inheritance (Griffiths and Shaw, unpublished data)

Striking differences in size and form of the teeth occur between strains

EXPERIMENTAL ANIMAL STUDIES OF ORAL TISSUE RESPONSES TO NUTRITIONAL AND METABOLIC VARIABLES

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The first recorded uses of experimental animals in dental research were concerned with the relationship of nutritional deficiencies to the oral tissues. In 1918 Mellanby[1] reported on the influence of fat-soluble A, later to be named vitamin D, on the calcification of the teeth of puppies, the rate of eruption, and the position of the teeth in the jaw. These studies were later extended to include correlations between animal and human investigations,[2] as well as extensive explorations of the influence of vitamin D on the rate of caries initiation in children[3-5] At about the same time as vitamin D's influence on calcification was recognized, Zilva and Wells[6] reported that the continously growing teeth of the guinea pig were the first organs to be influenced in experimental scurvy. Shortly afterward Hojer[7] reported that the microscopic changes in the teeth of scorbutic animals were sufficiently closely related to the amount of vitamin C in the diet to permit the development of a bio-assay for this vitamin. Wolbach and Howe[8,9] and Boyle *et al.*[10] studied the pathogenesis of the changes extensively and described the influences of scurvy on the odontoblasts and on the elaboration of collagenous intercellular substances. The influences of vitamin A on the ameloblasts, on bone growth, and on neural tissues also received intensive investigation in this early period.[11-13] Recent reviews of these and related investigations have been presented.[14,15] All these studies were limited to the use of histological and histochemical techniques, with little reference to the influence of these deficiencies on chemical composition or on susceptibility to disease. In contrast, the more recent investigations into the influence of fluoride ingestion on the calcified tissues have tended to emphasize chemical composition and susceptibility to caries.[16,17] These studies suggest the varied nature of the essential nutrients that cause variations in the structure and composition of the calcified tissues and also suggest the various types of response to be expected

During the same general period fragmentary information concerning the influence of the various nutrients, either singly or in groups, on the soft tissues of the oral cavity began to become available. Although good descriptions and photographic representations of the deficiency syndromes are available for man[18] and, to a lesser extent, for experimental animals,[19] there is still a great lack of detailed investigation in this area, especially with respect to careful histological and histochemical evaluations of the pathology involved in the syndromes. The greatest void is in our knowledge of the relationships existing among nutritional variations, metabolic disturbances, and periodontal structures in experimental animals, truly the no man's land of oral biology.

These brief introductory comments are intended to focus attention upon the need for multidisciplinary study of the many facets of these problems. Past studies indicate the relative specificity of individual nutrients and metabolic variables to influence targets among the oral tissues, which are

are under way in the same laboratory to check these relationships. The possibility that the ingestion of fluoride in the water supply at optimal levels may influence tooth size in human populations has been examined without conclusive results by Jarrett *et al.*,[35] whose data suggested that the caries-free teeth of children in some communities may be slightly smaller on the average than the carious teeth. Surveys of this type clearly indicate the problems associated with the collection and evaluation of data from children due to such factors as differing ethnic origins, dietary habits, and lengths of residence in a community.

Another example of the influence of nutrition upon tooth size has been obtained recently by Holloway in our laboratories during studies on the influence of a low-protein, high-carbohydrate diet on the offspring of rats (Holloway, Sweeney, and Shaw, unpublished data). A diet containing 8 per cent casein was fed for 2 months prior to pregnancy, and then throughout pregnancy and

Table 1

Influence of Variations in the Protein Content of the Diets of Rats During Pregnancy and Lactation on the Size of the Molar Teeth of Their Progeny*

		Mesiodistal diameter		Buccolingual diameter	
		Av reduction	t	Av reduction	t
Upper	M1	3 2	3.0	3 2	3.2
	M2	9 3	5.9	5 3	3.9
	M3	14.9	9.3	10 2	8.7
Lower	M1	3 4	4.0	8 1	6.1
	M2	3.7	2 8	7 0	5.5
	M3	15 8	13 0	8 5	7 1

* Values are expressed in terms of the average reduction in size of the molars of 20 progeny of the low-protein mothers in comparison with the size of the molars of 20 progeny of the normal mothers

lactation for 2 successive litters separated only by suitable rest periods on the same diet. Two other groups of rats were kept on diets containing 24 per cent casein and 72 per cent casein, the additional casein in each case being introduced in place of sucrose. The molars of the rats in the second litters of these females were measured for several dimensions. The greatest mesiodistal and buccolingual dimensions have been selected for discussion here. The differences in length of the molars of the rats from protein-deficient mothers and those of the rats from mothers on the diet containing 24 per cent casein are shown in TABLE 1. In all cases the teeth of rats from mothers fed the low-protein, high-carbohydrate diet were smaller than those of the controls. The percentage reductions for the second molars were greater than for the first molars; the percentage reductions for the third molars were greater than for the second molars. [illegible] were the third molars of rats from the protein-deficient [illegible] cent smaller than those of the controls, but also some [illegible] less prominent and even rudimentary. Preliminary studies [illegible] duction in size of the molars is due in part [illegible]

of the same species of rodents. Gove, Bailey, Sawin, and Shaw (unpublished data) observed that characteristic differences in the molar teeth of mice had appeared within seven generations of inbreeding mice from a mixed population. Some lines had a tendency toward supernumerary cusps. The size of the teeth and cuspal pattern varied significantly from line to line. Grainger *et al.*[30] also described differences in the size and morphology of the maxillary first molars of the Harvard caries-resistant and Harvard caries-susceptible strains of rats. The molars of the rats in the caries-resistant strain were significantly larger in the mesiodistal dimension than those of caries-susceptible rats. In addition, the mesial sulcus of the maxillary first molar of the rats in the caries-susceptible strain was shallower, narrower, and had more nearly parallel sides than the same sulcus in the caries-resistant strain.

Other studies have been conducted by Kifer *et al.*[31] with the Hunt-Hoppert caries-susceptible and caries-resistant strains of rats in which the widths of the fissures of the lower molars were measured. The fissures of the lower molars of the rats from the caries-susceptible strain were significantly wider than the corresponding fissures of the molars of the caries-resistant rats. It appears that this difference may be in the opposite direction from the differences for the Harvard caries-susceptible and caries-resistant strains; however, no such conclusion should be drawn without further study because different teeth were studied in the two surveys, as well as different sites for measurement, and they are difficult to relate quantitatively from one survey to the other.

Whether the physical differences between the teeth of caries-susceptible and caries-resistant strains in the two laboratories bear any causative relationships to the inherited susceptibility to tooth decay cannot be stated without much further investigation of a variety of strains with known predispositions to experimental dental caries. At present these observations can be interpreted only as interesting constitutional correlations. However, the fact that grossly detectable physical differences are conferred as a result of constitutional influences should warn us that a wide variety of more subtle differences in structure and composition also may be controlled in this fashion. Moreover, there is no reason to limit such constitutional controls to the tooth, for they undoubtedly have equal control over the entire physiological environment in which the tooth is maintained One excellent example of a chemical difference between rodent species has been provided by Sobel *et al.*, who observed striking differences in PO_4:$2CO_3$ ratios between the white rat and the cotton rat.[32,33]

In recent years the ability of nutritional factors to modify the size and morphology of teeth during their development has been demonstrated The first indication that the genetically ordained physical pattern in experimental animals could be modified came with the observation by Paynter and Grainger[34] that the administration to rats of fluorides, of a vitamin A–deficient diet, or of a diet with a strongly unbalanced calcium:phosphorus ratio through pregnancy and lactation resulted in offspring with smaller maxillary first molar teeth with narrower and shallower sulci than in offspring of the control females. No studies of the possible influence of these alterations in tooth form on caries susceptibility were included in this investigation. However, other experiments

of other lines can be maintained for long periods under the same dietary and environmental circumstances without appreciable evidence of the syndrome.

Interrelationship Between Oral Environment, Caries Activity, and Inherited Caries Susceptibility

The absolute requirements of suitable microbial flora and dietary components in the oral cavity for the initiation and progression of carious lesions in experimental rodents have been demonstrated under a variety of circumstances in recent years.[36] Keyes has suggested that caries inactivity in experimental hamsters may occur because the appropriate flora does not become established in the oral cavity.[37-39] Under such circumstances rodents from a strain that is inherently susceptible to the development of carious lesions can remain caries-inactive even when maintained on a highly cariogenic diet for an optimal experimental period. When hamsters from an unselected genetic background with respect to caries were maintained in relative isolation from caries-active hamsters but on a caries-initiating diet, they remained caries-inactive. On the other hand, maintenance of comparable hamsters on the same diet with hamsters that were caries-active resulted in a noticeable caries activity in the experimental subjects. Likewise, inoculating the oral cavity of the test animals with flora from feces of caries-active hamsters resulted in caries activity. As a corollary, penicillin-supplemented diets for pregnant, previously caries-active hamsters resulted in progeny that remained caries-inactive even though fed the caries-initiating diet. These findings give support to the hypothesis that dental caries is transmissible by nongenetic pathways. Where does inheritance fit into this hypothesis? Can apparently inherited caries resistance always be explained on the basis of a lack of appropriate inoculation when other conditions are met? Will appropriate inoculation always overcome any degree of apparently inherited caries resistance if appropriate diets are maintained?

We know that one or more microbial agents are necessary to initiate carious lesions. If dental caries is to occur, the oral cavity of the experimental subject that is free of microbial life up to birth must become inoculated with the appropriate microbiota, which must be able to establish themselves. We need to know to what extent such inoculations can contribute to apparently inherited characteristics of caries susceptibility. We also need to know whether the genetic constitution of an experimental animal can be oriented in such a way as to resist caries initiation even when inoculation occurs repeatedly.

My associates and I desired to answer two questions that arise logically from this concept: (1) Will offspring from a strain presumed to be caries-resistant by reason of generations of selection to emphasize a low rate of caries activity become caries-active when maintained in close contact with caries-active rats from a strain presumed to be caries-susceptible by reason of selection to emphasize a high caries activity? (2) Will progeny of a presumably caries-susceptible strain remain caries-active when maintained only with representatives of the presumably caries-resistant strain? The very nature of the selection process to obtain a caries-resistant strain tends to minimize gross opportunities for the offspring to come in contact with caries-active animals. In the Harvard caries-resistant strain, on the average only one caries-active rat of

at the amelodentinal junction and not solely to a thinner enamel. The molars of the offspring of the mothers fed the low-protein, high-carbohydrate diet were much more susceptible to tooth decay than were those of the offspring of the control rats (TABLE 2). Furthermore, the third molars of the experimental rats erupted on an average of 5 days later than did those of the controls. On the basis of the present study it is not possible to know the exact agent responsible in the progeny for these changes in tooth size, eruption time, and caries susceptibility. The actual protein deficiency of the mother's diet may have been buffered to some extent by the maternal organism to protect the progeny. The ultimate cause of the above alterations may have been a secondary caloric restriction by reason of reduced milk flow or some other secondary influence.

Many more studies must be conducted in this area before the relationships will be fully understood. At present it is clear that the variation of certain nutrients during tooth development can turn both their physical characteristics and their susceptibility to tooth decay away from norms that would be predicted from the genetic constitution. However, these influences can be achieved without such gross teratogenic phenomena produced by gross deficiencies as those described by Kalter elsewhere in this monograph. From other studies, the influence on chemical composition of fluoride ingestion and of variations in the dietary calcium:phosphorus ratio (Sobel *et al.*[33]) has been clearly demonstrated. It is interesting that both the fluoride supplementation and the low-protein, high-carbohydrate ration resulted in smaller teeth whereas the influence on caries susceptibility was in the opposite direction for the two treatments

TABLE 2

INFLUENCE OF VARIATIONS IN THE PROTEIN CONTENT OF THE DIETS OF RATS DURING PREGNANCY AND LACTATION ON THE CARIES ACTIVITY OF THEIR PROGENY

Group	No of rats	No of carious molars			No of carious lesions			Extent of carious lesions		
		Av	SEM*	t†	Av	SEM*	t†	Av	SEM*	t†
Control	13	4 1	0 7		5 8	1 2		9.1+	2 4+	
High protein	13	1 2	0 5	3 6	1.8	0 9	2 7	2 3+	1 2+	2.5
Low protein	23	6 7	0 4	3 0	11 3	0 3	4 4	30 6+	4 0+	4 6

* Standard error of mean

† Lines connect values being compared for Fisher t test of significance

Genetic variations in susceptibility to periodontal disease also have been demonstrated. The wide variation in susceptibility between species is well known, strains of the hamster and the rice rat are in general susceptible, while various strains of the white rat tend to be resistant However, within species striking variations in susceptibility are prevalent. This variation has been observed with great frequency in our laboratories in the rice rat; some family lines have high susceptibility to the periodontal syndrome, while the offspring

few hours of age would have the same environmental opportunity to establish an oral flora compatible with caries initiation and progression as the caries-susceptible offspring. Likewise, in the fourth group the caries-susceptible offspring would have the same environment for the establishment of an oral flora as the caries-resistant offspring.

The caries-resistant rats of the first and second groups were on experiment for 140 days and remained caries-inactive regardless of the caries activity or inactivity of their foster mothers. In contrast, the genetically caries-susceptible offspring in the third and fourth groups all had developed a high rate of caries by the eightieth day, irrespective of the state of caries activity of their foster mothers. No difference in rate of caries activity was demonstrable between the first and second or between the third and fourth groups. Hence caries initiation was independent of the genetic background of the nursing mothers and parallels the rate of caries initiation anticipated from the genetic characteristics of their parents.

Another test of the same general type has been reported by Hall and Shaw,[41] who used parabiotic pairs consisting of a caries-susceptible and a caries-resistant rat. The rate of caries initiation in each rat was characteristic of its strain, no rat influencing the rate of caries initiation of his mate.

From these studies it can be concluded that under the environmental circumstances described, offspring of a caries-susceptible strain are equally able to establish suitable flora for the initiation of carious lesions whether maintained in relative isolation from older members of their own strain or in close contact with them. Likewise, offspring of the caries-resistant strain remain caries-inactive when brought into close contact with members of the caries-susceptible strain at various periods It seems inconceivable under these circumstances that the offspring of the resistant strain have not had adequate exposure to the microbial agents necessary for the initiation of experimental caries under the dietary conditions offered.

This assumption is especially reasonable in view of the following facts: the two strains, kept in the same room for ten years, are maintained on the same diet and fed on the same schedule by the same technician; ration cups, water bottles, and cages are used interchangeably for the two strains and are only washed, without any attempt at sterilization, between use by rats of different strains. With all these opportunities for the establishment of an appropriate oral flora, the rats from the caries-resistant strain remain caries-inactive for prolonged periods. Whether this resistance is due to anatomic or structural characteristics of the teeth that make them less suitable for the initiation and progression of carious lesions in the presence of an appropriate oral flora, or is due to metabolic influences operating through the saliva or the blood that inhibit the establishment of an appropriate flora in the oral cavity cannot be determined. However, we may conclude that the selection of breeding stock over the years under the experimental circumstances prevailing in our laboratory has led to the development of a strain with unique characteristics of resistance to tooth decay; a similar procedure has led to the establishment of a caries-susceptible strain that is dramatically different with respect to the initiation and progression of carious lesions under comparable environmental conditions. On the basis of our present knowledge about these two strains,

breeding age in every two or three generations has been found in the colony in recent years. The reverse is true in the Harvard caries-susceptible strain, in which no rat used for breeding in the past five years did not have grossly detectable carious lesions weeks prior to mating.

Since we have been maintaining for the past decade strains of rats that have been selected generation by generation to emphasize either a high caries activity or caries inactivity, we have sought to answer these questions. The experimental subjects in these investigations have been representatives of our two most divergent strains with respect to caries initiation and progression which, for the sake of simplicity, we shall refer to as the Harvard caries-resistant and Harvard caries-susceptible strains. These descriptive terms have been used in our laboratory since the beginning of our efforts to develop such strains. In actuality, the terminology could be totally incorrect if their final caries incidence were a resultant of the one strain having an inadequate inoculation of the appropriate microbial flora and of the other strain having an early and overwhelming inoculation from their mothers.

All subjects in the following experiments were maintained on our usual cariogenic diet of 700 + 15 per cent Cellu flour,[40] the diet used without interruption for maintenance of the breeding colonies of both the above strains generation after generation.

In the first half of the first experiment, pairs of weanling rats composed of a caries-resistant and a caries-susceptible animal of the same sex were maintained in wire-bottom cages Suitable littermate controls for both the caries-resistant and the caries-susceptible strains were kept singly in cages and in pairs of the same strain and sex in other cages After eighty days the caries-susceptible rats maintained alone, those in pairs with their littermates, and those in pairs with caries-resistant rats had almost identical incidences of carious lesions. After two hundred and fifty days the caries-resistant rats maintained in the same three caging conditions showed no evidence of carious lesions.

In the second half of this experiment, caries-resistant weanlings were maintained in cages with young adults of the caries-susceptible strain who had already developed a number of rapidly progressing, grossly visible carious lesions Caries-resistant littermates were maintained singly in comparable cages After two hundred and fifty days there was again no evidence of caries activity among the offspring of the caries-resistant strain, whether housed with a caries-active rat or alone

In the second experiment of this series, half of the offspring of a group of caries-resistant females bred by caries-resistant males were taken from their mothers within twenty-four hours after parturition to be fostered by caries-susceptible females; half of the offspring of a group of caries-susceptible females bred by caries-susceptible males were taken from their mothers within twenty-four hours after parturition to be fostered by caries-resistant females. Thus there were four groups of offspring: (1) caries-resistant offspring reared by their own mothers, (2) caries-resistant offspring reared by caries-susceptible mothers; (3) caries-susceptible offspring reared by their own mothers; and (4) caries-susceptible offspring reared by caries-resistant mothers. The assumption in this experiment is that the caries-resistant offspring in the second group from a

permanent molars to the high level of 130×10^{-7} per cent of injected dose per milligram of the layer of calvarium adjacent to the dura mater.

In contrast, there was a much greater range in uptake for radiophosphorus, typical of the second group of elements; it varied according to the tissue's structure, age, and degree of mineralization and according to the depth at which samples were removed.[43] The highest specific activity noted was in the alveolar bone of the mandible, where a value of 6750×10^{-8} was noted in the layer adjacent to the periosteum; the lowest specific activity was observed in the internal enamel of erupted molars, where the value was 1×10^{-8}. The radiophosphorus uptake was high in the enamel of unerupted teeth, comparable to the levels in the bones. Regardless of the tissue, the radiophosphorus gradients increased toward the layers that were in closest proximity to the adjacent tissue fluid, whether it was connective tissue fluid or saliva. Again, when the outer surface of enamel was protected from contact with saliva there was a striking reduction in the uptake of radiophosphorus in all layers of the enamel, suggesting that saliva was the primary source of radiophosphorus for the enamel.

In studies of the calcified tissues of rats and monkeys, an increase in sodium content with age has been shown (Bogoroch, Yen, Shaw, and Sognnaes, unpublished data). When these animals were maintained for prolonged periods on sodium-deficient diets, the sodium contents of the calcified tissues were maintained at levels comparable to those in young animals, whereas the levels in the controls increased to concentrations consistent for the particular species. When acidosis was produced and maintained in rats by the intraperitoneal injection of NH_4Cl, withdrawal of sodium in detectable amounts from the enamel and dentin, as well as from the bones, occurred. Thus radiosodium may be considered a typical tracer for the third group of elements. In studies with radiosodium in monkeys and rats, the ratio of specific activity of radiosodium in the calcified tissue to the specific activity in the blood has been shown to vary according to the tissue (Bogoroch *et al*, unpublished data). This ratio, which is indicative of the exchangeability of the sodium within a tissue, was lowest for the enamel at about 1:6 other ratios were. dentin 1:3 and bone 1:2 in comparison with 1:1 for the soft tissues. The sodium in the calcified tissues of older animals was less readily available for exchange than that in young animals.

The use of radiocalcium in monkeys has demonstrated the low rate of exchange of calcium in the calcified tissues under normal metabolic conditions (Yen and Shaw, unpublished data). After 168 hours, the ratios of specific activity in the tissues to specific activity in the blood were. endosteal layer of the calvarium suture, 1:15; calvarium, 1:25; enamel of unerupted teeth, 1:30, shaft of long bones and internal dentin of unerupted and erupted teeth, 1:50, dentin of erupted teeth, 1:500, and enamel teeth, 1:1000.

There was an exchange gradient for radiocalcium in bones, the subperiosteal layer exchanging more rapidly than the inner layers in long bones; the exchange was reverse in the flat First and second molars showed an increasing gradient from the internal toward the surface enamel and a marked decreasing gradient from internal dentin toward external dentin. There was also an increasing gradient in similar layers from the 5-hour samples toward the 168-hour samples.

These relationships of the type of tissue, its age, and the metabolic conditions

the use of the terms caries-resistant and caries-susceptible appears to be justified.

Rosen and his co-workers at Michigan State University, East Lansing, Mich., where the pioneer studies on the development of caries-resistant and caries-susceptible strains were conducted, have also tested the effect of fostering offspring of one strain by mothers of the opposite strain (unpublished data). In order to disturb the young as little as possible, mothers from the caries-resistant strain were removed from their litters as soon after parturition as possible and were placed with newly born litters of the caries-susceptible strain. The mothers of the caries-susceptible litters were placed with the caries-resistant litters. Under these circumstances, when the Hoppert-Webber-Canniff type of diet[42] was used instead of the purified diet used in the Harvard experiments, the offspring again developed the incidence of carious lesions expected for their genetic background and different from that of their foster mothers.

From these studies it is clear that constitutionally inherited factors are important in the determination not only of the morphology and eruption of teeth, but also of their caries susceptibility. However, we must not overlook the fact that such inherited influences may be modified by the superimposition of another influence such as the absence of an appropriate oral flora or the maintenance of the subject on a caries-inhibiting diet, either of which procedure may prevent the full expression of the genotype.

Relation of Tissue, Age, and Metabolic Condition to Mineral Exchange

The calcified tissues of the oral cavity provide interesting opportunities to study mineral exchange under varied circumstances and often with fewer morphologic variables than exist in other areas of the skeletal system. The enamel and dentin of erupted and functioning teeth, in particular, offer simpler conditions for these studies than do other calcified tissues because of the total absence of cellular remodeling and of growth and deposition of new crystallites except in the secondary dentin. The elements for which radioisotopes are available for use in studies of the mineral exchange, after calcification of enamel and dentin is complete, fall into three general categories: those that are normal components of the body fluids, but are not significant components of the inorganic moiety of the calcified tissues; those that are significant components of the calcified tissues, but cannot be withdrawn without cellular activity, and those that are significant components of the inorganic portion and can be partially withdrawn or replaced during metabolic variation without cellular remodeling.

When radioiodine was used as a tracer that was typical of the first group of elements, there was a limited and relatively similar uptake in all the mesenchymal hard tissues, with no conspicuous gradients from one layer to the other.[43] The enamel of unerupted and erupted teeth had especially low counts, and there was no striking difference between the internal and the external layers of dentin despite the active deposition of secondary dentin. When the outer surface of the enamel was covered to prevent contact with the saliva, the level of radioactivity throughout all layers of the enamel was greatly reduced. The greatest range in radioiodine uptake occurred from the low level of 1×10^{-7} per cent of injected dose per milligram of internal enamel of fully erupted first

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of the host to mineral exchange in the calcified tissues should serve as an indication of the plentiful opportunities for research that exist in this area.

Summary

In a very real sense the hard tissues of the oral cavity are kymographs that record many facts about the metabolic conditions that prevailed at the time of their formation. The first readings of these kymographs were made primarily by visual evaluation of histological preparations. To this one must now add evaluations of their ultrastructure, measurements of the size and gross morphology of the teeth, the chemical composition of the enamel and dentin, and the susceptibility to oral disease of the teeth and their supporting structures. Possibly above all other factors, one must be certain of the quality and suitability of the animals chosen as subjects for any investigation. Furthermore, experimental designs must be planned with the greatest care in order that valid interpretations be made of the data obtained.

The challenge of the future in the study of the nutritional and metabolic variables that influence the oral tissues, particularly those that are mineralized, can be seen from the varied interrelationships that impinge upon them, examples of which are presented in this introduction to the area. Throughout this monograph, other reviews will be found that elaborate upon specific portions of this general area and that demonstrate the encouraging broadening of the horizons since the early pioneering observations of three and four decades ago.

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often necessary. At times the clinician must pursue a symptom. Often it is difficult for the patient to decide which of his complaints is the more serious or distressing. For example, the clinician may find it necessary to elicit more data concerning progressive weight loss, unemphasized by the patient, and to devote less attention to the patient's primary concern with insomnia. As in medicine, the dentist must use the history, both dental and systemic, the clinical examination, and the laboratory data to make a diagnosis (Macbryde, 1957).

Because of the similarity of oral tissue changes seen in conjunction with various systemic alterations and because of the infrequency of pain associated with oral disease, the dentist finds it difficult to group oral symptoms together to form quickly recognizable complexes or syndromes. Every physician knows that "dermatitis, diarrhea, and dementia" means pellagra and that "tremor, tumor, and tachycardia" indicates hyperthyroidism but, due to the special cytoarchitecture of the teeth and periodontium, the oral symptoms and signs of systemic disease are so modified that they alone do not allow the dentist to group them accurately into oral syndromes.

It must also be borne in mind that, although an associated systemic disease may have been established, this does not necessarily mean that the disease in question has a cause-and-effect relationship (Karshan, 1952). Rather, it may influence the local factor in the causation of the oral disease. On the other hand, it may bear direct influence: for example, the disease scleroderma (Stafne and Austin, 1944), which may cause degenerative changes in the periodontal ligament (FIGURE 1).

Local Intraoral Factors

Many investigators have demonstrated that in the absence of abnormalities of intake, absorption, or systemic metabolism, local factors per se may modify tissue demands and local metabolism to such an extent that lesions are created in an otherwise healthy organism. In this connection, consideration must be given to the limitations of laboratory techniques. Systemic changes may occur in otherwise healthy individuals with periodontal lesions but may be so subtle that present-day laboratory methods will not detect them. However, excellent clinical results have been obtained over the years in these cases by only local therapeutic procedures (Goldman *et al.*, 1956, 1959), and it is fairly certain that local irritants have been responsible for the lesions (FIGURE 2).

Moreover, the role of calculus, materia alba and mucin plaques, tooth anatomy and position, food impaction and retention, inadequate restorative work, and oral sepsis in initiating gingival lesions is well known (FIGURE 3). In the tooth attachment apparatus the changes resulting from occlusal traumatism are also examples of the effects of local intraoral factors. In studying the characteristics of periodontal disease one is impressed with the chronicity of the lesion. Since the causative agent is constantly present, complete healing cannot take place and a chronic inflammatory or degenerative process is thus set up despite any attempt by the repair process.

Consideration must be given to oral lesions that begin locally and mechanically and affect the general health of the patient. The literature often calls attention to disease of the gingiva, following mechanical manipulation of the

CLINICAL OBSERVATIONS ON THE MODIFICATION OF HUMAN ORAL TISSUE METABOLISM BY LOCAL INTRAORAL FACTORS

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The uniqueness of the oral tissues, from the standpoints of anatomic relationships and local environment, has aroused the curiosity of many investigators of organ systems. There exists an overwhelming variety of intraoral influences capable of triggering the complex reactions resulting in changes in oral tissue metabolism, especially for one beginning the study of oral disease. The picture is additionally complicated by systemic factors which are also capable of affecting the metabolism of oral tissues. This discussion will attempt to depict the delicate interplay of local and systemic etiological factors and the resultant clinical manifestations of certain oral diseases.

Characteristics of Oral Diseases

Cheraskin (1959) and others have pointed out that health and disease seldom, if ever, become clinically evident in pure black and white—that between the two there is an infinite number of shades of gray, a limitless gradation from health to sickness. The term subclinical disease is used to connote microscopic and biochemical pathological changes of insufficient severity to be detected clinically (Glickman, 1958). In addition to the necessity of recognizing relative states of sickness and health, one is confronted with another characteristic of oral disease: that a pathological process may be the end product of more than one causative factor.

Diseases of the periodontal structures are classic examples of causation by multiple etiological agents Local etiological factors are those in the immediate environment of the teeth and supporting structures; systemic causative factors refer to systemic conditions of the patient that influence the metabolism of periodontal tissues In certain cases several local irritants may produce a periodontal lesion in an otherwise healthy individual; in others the clinical picture of disease is the result of the interaction of local trauma and an appropriate systemic substrate. However, it is difficult to evaluate and quantitate the systemic relationship to periodontal disease because of the many complex reactions occurring in the periodontium when excess stress and chronic inflammation coexist. In many instances changes in the tissues of the oral cavity and periodontium are manifestations of early disease elsewhere. However, changes taking place in the gingivae are rarely, if ever, specific for any complicating systemic disease; they exhibit the same characteristics of tissue change as are seen in local phenomena and thus are described as edematous, suppurative, hyperplastic, atrophic, hemorrhagic, or necrotic Oral symptoms are apt to appear some time before any other striking physical signs of systemic disease appear and before laboratory tests can detect a disordered physiology A detailed and accurate history is thus essential, and careful cross-questioning is

often necessary. At times the clinician must pursue a symptom. Often it is difficult for the patient to decide which of his complaints is the more serious or distressing. For example, the clinician may find it necessary to elicit more data concerning progressive weight loss, unemphasized by the patient, and to devote less attention to the patient's primary concern with insomnia. As in medicine, the dentist must use the history, both dental and systemic, the clinical examination, and the laboratory data to make a diagnosis (Macbryde, 1957).

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FIGURE 1. Radiogram showing widening of periodontal membrane space in scleroderma.



FIGURE 2. Periodontitis before and after treatment Note in the upper photograph the heavy supragingival calculus before therapy and in the lower picture the gingival health restored with local therapy.

teeth, as a souce of bacteremia. Manipulative procedures have been described as the most common cause of the recurrence of episodes of subacute bacterial endocarditis. Recognition of the potential danger from bacteremia in patients with periodontal disease requires the inclusion of chemotherapy before scaling



FIGURE 3. Upper photograph shows gingival inflammation about the mandibular right lateral and cuspid region. This area can be compared to that seen on the left side. The position of these teeth is directly related to the causation of this process In the lower picture there is also gingival inflammation associated with crowded incisors Note the frenum attachment high on the gingival margin of the mandibular left central incisor

or surgical procedures in patients with a history of rheumatic fever or heart valve disease. Other than these studies on periodontal disease and bacteremia, there is a lack of definitive information on other systemic disturbances that may result from periodontopathies in humans. Isolated reports in the literature indicate that some interrelationship may exist, but the lack of standardized criteria limits the value of these observations.

An oral lesion initiated by local factors is a form of perlèche or pseudoariboflavinosis (Ellenberg and Pollack, 1942). In this condition there is a decrease in vertical dimension and loss of the normal intermaxillary space. Such a cheilotic lesion may be deeply fissured and may exhibit spontaneous remissions and exacerbations. Burket (1952) points out that lesions associated with a decreased intermaxillary space slant downward and outward, whereas lesions of angular cheilosis associated with ariboflavinosis are more nearly horizontal. Such lesions are found more commonly in edentulous elderly patients, in pa-

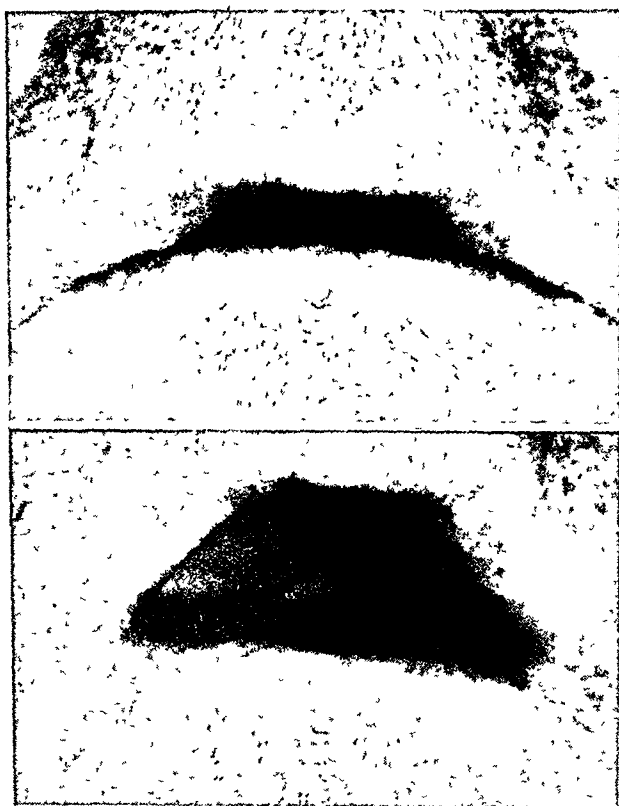


FIGURE 4 This patient was admitted to the hospital with severe nutritional deficiency Intensive therapy resulted in regression of all the lesions except the cheilosis Insertion of dentures resulted in amelioration of the cheilosis (Courtesy of Lester W Burket)

tients who have not received frequent denture servicing or whose teeth have been severely abraded The elimination of etiological factors by the construction of dentures that restore the vertical dimension and also increase the masticatory efficiency results in healing of these lesions (FIGURE 4). The angular cheilosis of riboflavin deficiency extends a few millimeters from the angles of the mouth on to the cheek mucosa This is not observed in pseudoariboflavinosis Here, too, it may be noted how a locally induced lesion may produce more serious consequences for the total organism Because of a lack of dentures the patient may voluntarily restrict his vitamin- and protein-containing foods, preferring soft foods that are rich in starch and carbohydrates Such nutritional imbalance may lead to angular cheilosis due to the deficiency state.

Thus similar oral lesions may have either local or systemic causation; on the other hand, a condition initiated by local factors may result in systemic disturbances.

Interaction of Local and Systemic Causative Factors

The remainder of this discussion will concern alterations in oral metabolism that result from the combined effects of local and systemic etiological factors. As has been mentioned, it is extremely difficult to assay the proportionate role of each factor responsible for the observable evidence of disease (Person, 1955). This is due partly to lack of information concerning systemic diseases and partly to the paucity of data regarding the interactions of local and systemic factors. Since this presentation was intended to report clinical observations of the modification of human oral tissue metabolism induced by local intraoral factors, we selected cases in which systemic and local factors were present. The systemic factors were then regulated and the resultant oral changes described. Those changes resulting from the elimination of the local agents then were documented.

Endocrine Disorders

Because it may involve many organs, an endocrinopathy is usually not a clear-cut pathological entity; some of the fundamental chemical processes modified by hormonal action may be common to many body tissues. Since the ultimate manifestation of disordered hormone secretion is reflected in abnormalities of cellular function, it is possible for disturbances in mechanisms or essential cell constituents, or for genetic or structural abnormalities of the cell, to simulate endocrinopathies. Endocrinological disease offers a challenge to the dentist and physician in that it combines specific signs with measurable metabolic and hormonal changes Furthermore, many of the endocrine diseases are amenable to substitution therapy, and in some cases a permanent cure is effected. In the last analysis, the diagnosis of endocrine disease is made by combining clinical experience with evaluation of laboratory tests, because either alone is insufficient for an adequate appraisal of the patient (FIGURES 5 and 6).

Gingival lesions in young females of postpuberty age have been described (Ziskin and Silvers, 1943). Various degrees of gingival enlargement have been found and range from firm, well-attached, hyperplastic tissue to discolored, retracted, grossly inflamed, and markedly hyperplastic tissue. Laboratory examination often discloses a lowered basal metabolic rate (20 to 40 per cent below normal) and an elevated serum cholesterol (300 to 700 mg./100 ml.). A female 14 to 20 years of age or older may not have started menstruation We are informed by physicians that this condition of hypothyroidism is not uncommon. In such cases the gingival margin has not receded, the edge of the tissue being at the height of the contour of the crown of the tooth Persons so affected exhibit an inflammatory change similar to that seen in eruptive gingivitis. The secondary tissue changes are distinctly similar to those due to local environmental irritation. The influence of a lack of menses is difficult to evaluate. FIGURE 7 shows the mouth of a 14-year old girl who presented with marked inflammatory hyperplasia characterized by redness, retraction, and a bulbous appearance of the interdental papillae. The dental history disclosed that the teeth had always been covered by gingiva. Medical examination re-

vealed that the patient had a low basal metabolism, −32, and that she as yet had not had any menses. Her physician administered 2 gr. of thyroid daily; the photograph taken afterwards (FIGURE 8) reveals the change in oral tissues in 3 months. The marked enlargement has been reduced, but local irritations of calculus and food impaction have kept the gingiva inflammed.

The incidence of gingival changes during pregnancy varies with the reports, the average being about 50 per cent. Gingival enlargement in pregnancy may



FIGURE 5 A case of marked gingival hyperplasia affecting the gingivae around all the teeth in a 14-year-old boy. The medical history disclosed the following partial cryptorchidism, undeveloped genitalia, possible pituitary disease, and mild hypochromic anemia Systemic therapy consisted of three courses of anterior pituitarylike substance, a regimen with which remarkable development was noted in the genitalia

FIGURE 6 Same case Periodontal therapy essentially consisted of gingivectomies and oral physiotherapy. Although gingival healing was moderately slow and retarded, a favorable result was obtained Orthodontic therapy was then instituted

FIGURE 7. Gingival inflammation in a young girl with low basal metabolism.

FIGURE 8 Same case after systemic regulation with thyroid medication Note reduction in inflammation, however, local irritants are still responsible for inflammatory changes noted interdentally

appear as a generalized hyperplasia characterized by discoloration, retraction, bleeding, and a "mulberry" surface (Ziskin *et al.*, 1933). Although the gingival changes attributed to pregnancy are usually only accentuations of a prior inflammatory process, occasionally they are found in a person who had exhibited no notable gingival alteration before becoming pregnant, and a gingival enlargement may be observed in an area entirely free of local environmental irritation. The interdental papillae are bulbous occasionally simulating a discrete tumor like mass (FIGURES 9 and 11). The surface is of interest. in some instances it is smooth and glistening and in others it is rough-textured and ma-



FIGURE 9 Enlargement of gingiva associated with pregnancy This was the seventh month of the first pregnancy

FIGURE 10 Same case This is the sixth month of the second pregnancy. The dentist removed the bridge but food impaction was still present between the cuspid and bicuspid as well as the cuspid lateral area

FIGURE 11 Same case This is the eighth month of the second pregnancy The inflammatory lesions have returned There were no other lesions of this type elsewhere in the mouth

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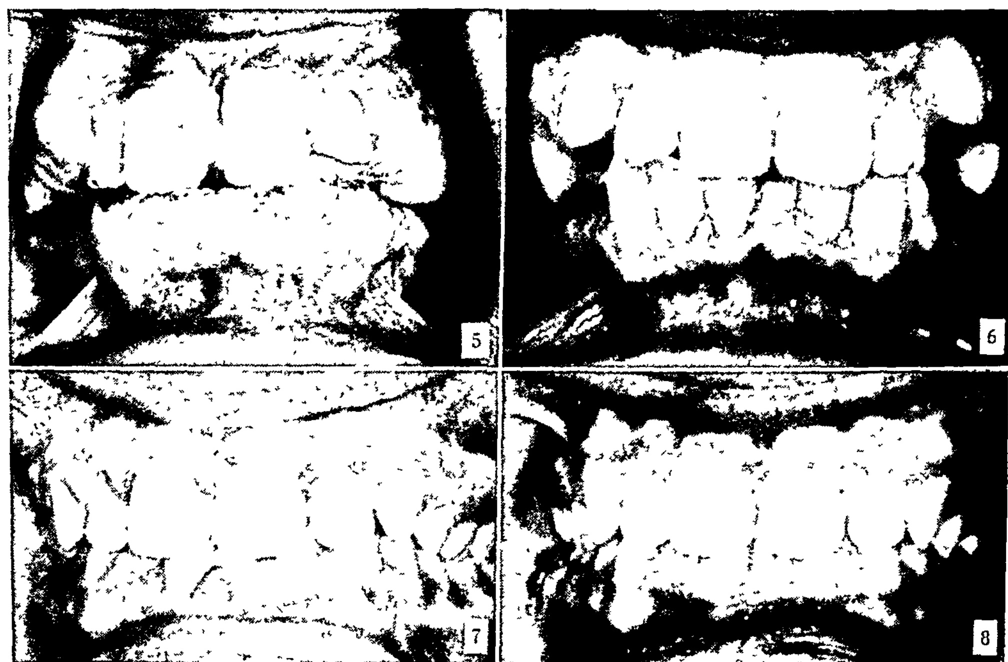


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genta in color. The clinical picture changes as pregnancy progresses, usually reaching a highest point of severity at the seventh or eighth month. Microscopically, endothelial proliferations with capillary formation associated with inflammatory changes constitute the main feature; this vascular change accounts for the clinical picture (Maier and Orban, 1949). A spontaneous reduction in size of the gingival hyperplasia often follows the termination of pregnancy, and in some mild cases a complete remission is experienced. Usally, however, a residual inflammatory lesion persists and should be treated (FIGURES 12 to 14).

The oral tissues often reflect the hormonal imbalance of the female climacteric. At this time there is not unusually a sore and tender mouth, and the



FIGURE 15 Gingival inflammation associated with menopause. Lower photograph taken after patient was placed on stilbestrol, shows gingival tissue response to local therapy.

common complaint of burning or dryness of the oral mucosa. The cheek mucosa at times has a milky appearance and irregular grayish white areas that may be mistaken for leukoplakia. There is a diminimished keratinized protective covering of these tissues. A gingival inflammation localized at the free and attached gingiva and fiery red in color is not uncommon. Gingival changes identical to those described as chronic desquamative gingivitis have also been observed; such lesions are characterized by diffuse inflammation of the gingiva the epithelium of which tends to peel away from the corium. Sometimes bullae precede the desquamative process.

FIGURE 15 shows the oral picture of a 61-year-old woman having many of the characteristic signs and symptoms of menopausal changes. The gingival tissues were bright red, hyperplastic, and retracted from the tooth surfaces. Periodontal therapy was instituted to remove the calculus deposits and there was considerable healing of the gingivae. However, the discoloration and the







FIGURE 12 Gingival inflammation observed during pregnancy No therapy was instituted in this case until after termination of the pregnancy

FIGURE 13 Same case Note reduction of inflammation after parturition No therapy had been instituted

FIGURE 14 Same case Note tissue changes after scaling, subgingival curettage, and oral physiotherapy.

dent. While tooth mobility was not too pronounced, there was a certain dullness to percussion and a very slight movement to finger pressure. Radiographs revealed a marked bone destruction with advanced loss of the marginal area. No special widening of the periodontal membrane space nor loss of remaining lamina duia could be discerned (FIGURE 18).

The differential diagnosis rested between a marked gingival inflammatory process with underlying bone resorption and with the patient's inability to overcome the lesion (poor resistive and reparative factors) and a disease process of the attachment apparatus of the teeth (periodontosis) with secondary gingival inflammation. FIGURE 17 shows the appearance of the gingival tissues at the initial examination. The patient stated that this condition had begun 5 years previously when he was given orthodontic appliances for malaligned



FIGURE 17. Severe overgrowth in a 15-year-old male who had gingivectomies performed twice. Lower photograph: after regulation of systemic disorder Local therapy was instituted and the healing may be seen

teeth. Three years before this examination and one year previous he had undergone gingivectomies for the gingival hyperplasia in the anterior portion of the mouth; the overgrowth returned within a short time postoperatively

A complete medical evaluation was suggested. The patient was placed on the medical service of the hospital for one week and the following data were collected.

Urine was negative except for a low specific gravity, but after a concentration test on the seventh hospital day the urine specific gravity was 1.030. Hemoglobin was 14.5 mg. The white blood count was 8,800, with a normal differential, and the smear was normal. The stools were negative for occult blood. N.P.N., 28 mg.; T.P., 7.9 gm.; albumin, 5.5 gm.; globulin, 2.4 mg.; Hinton, negative. On the second hospital day, the calcium was 10.9 mg. phos. 2.4 mg , alkalıne phosphatase, 2 2. On the fourth hospital day the calcium was 10.9 mg.; phosphorus, 3.2 mg.; and ionized calcium, 4.3. Urine Sulkowitch

edema did not disappear, nor did the bleeding halt despite the thorough home care of the patient The patient continued to have other symptoms of the menopausal syndrome, and her physician placed her on daily doses of diethylstilbestrol. The hot flashes and other symptoms disappeared and the gingival tissues became considerably firmer and pinker. This case illustrates a gingival manifestation of a systemic alteration that was treated locally, first with limited

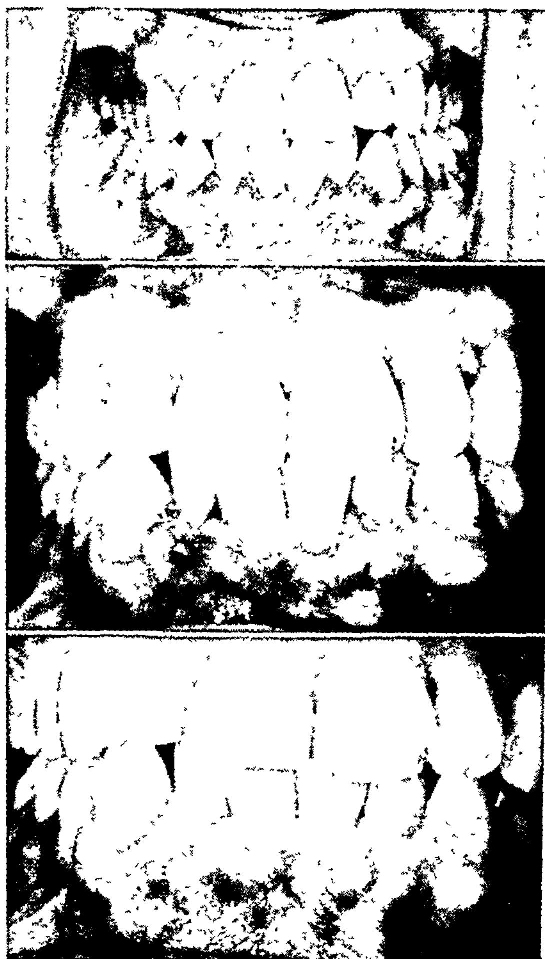


FIGURE 16 (*Top*) Hormonal gingivitis (*Middle*) Changes associated with hormona therapy only (*Bottom*). Healing after scaling and curettage

success, but finally with good results when the systemic background was corrected. (*See* FIGURE 16.)

An interesting systemic condition that has been difficult to define but probably should be considered as an endocrine abnormality is demonstrated in the following two cases.

Case 1. A 15-year-old male presented with a marked inflammatory gingival hyperplasia with retraction of the gingival tissues from the tooth surface Bleeding was pronounced, and the gingival color was reddish pink (FIGURE 17) Stippling of the surface had entirely disappeared, and deep pockets could be probed about every tooth surface; some visible areas of suppuration were evi-

the dietician could provide, and was discharged, to be followed in the Endocrine Clinic as an outpatient.

The altered glandular activity was regulated and then local therapy of the periodontal lesion was initiated. The treatment consisted of removal of all calculus deposits, elimination of pockets by gingivectomy, instruction in oral physiotherapy, and occlusal adjustment by means of selective grinding. The radiograms taken 2 years postoperatively revealed healing of the alveolar crests,

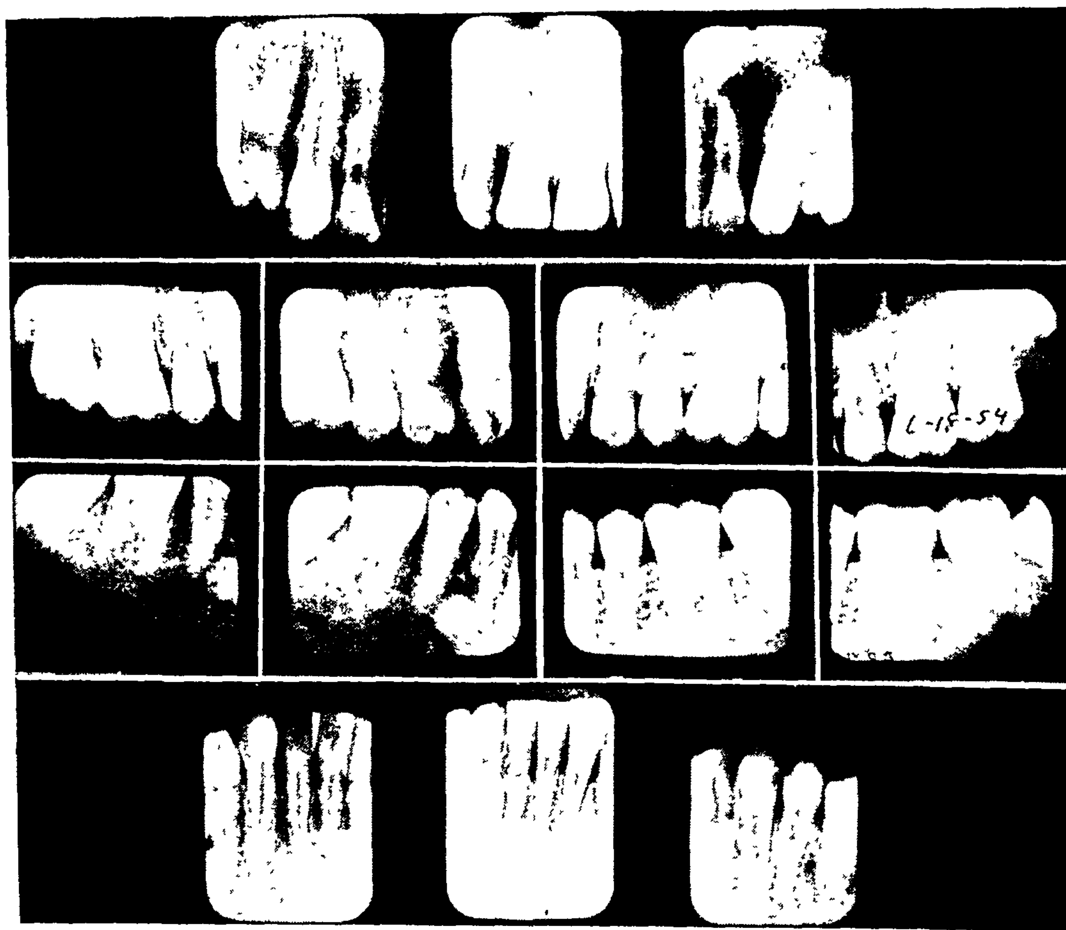


FIGURE 19. Radiograms (see FIGURES 17 and 18) 3 years after therapy was completed. Areas of healing are noted throughout the mouth

with the formation of a cortical layer and also a suggestion of osseous regeneration. The gingival tissues were pink, stippled, and adhered to the teeth (FIGURES 19 to 22).

This case illustrates the adverse effect of a systemic abnormality on local therapeutic procedures and the dramatic healing that occurred after the systematic alteration was controlled and further local treatment instituted

Case 2 A 16-year-old female presented herself with a marked gingival inflammation throughout her mouth Bleeding and retraction of the reddish edematous gingival tissues were most marked in the anterior regions (FIGURE 23) Pocket depth was present around all of the teeth. Tooth mobility was slight in the posterior regions and moderate anteriorly. Radiography re-

was 2 plus on three determinations and 3 plus on one. Twenty-four–hour urine phosphorus excretion was 181.5 mg. on the third hospital day and 448.2 mg. on the sixth hospital day. Twenty-four–hour calcium excretion in the urine was 136 4 mg. on the third hospital day and 167.6 mg. on the sixth hospital day. P.S.P., 100% excretion in 60 min. Basal metabolism rates were −22, −19, −16, and −20.

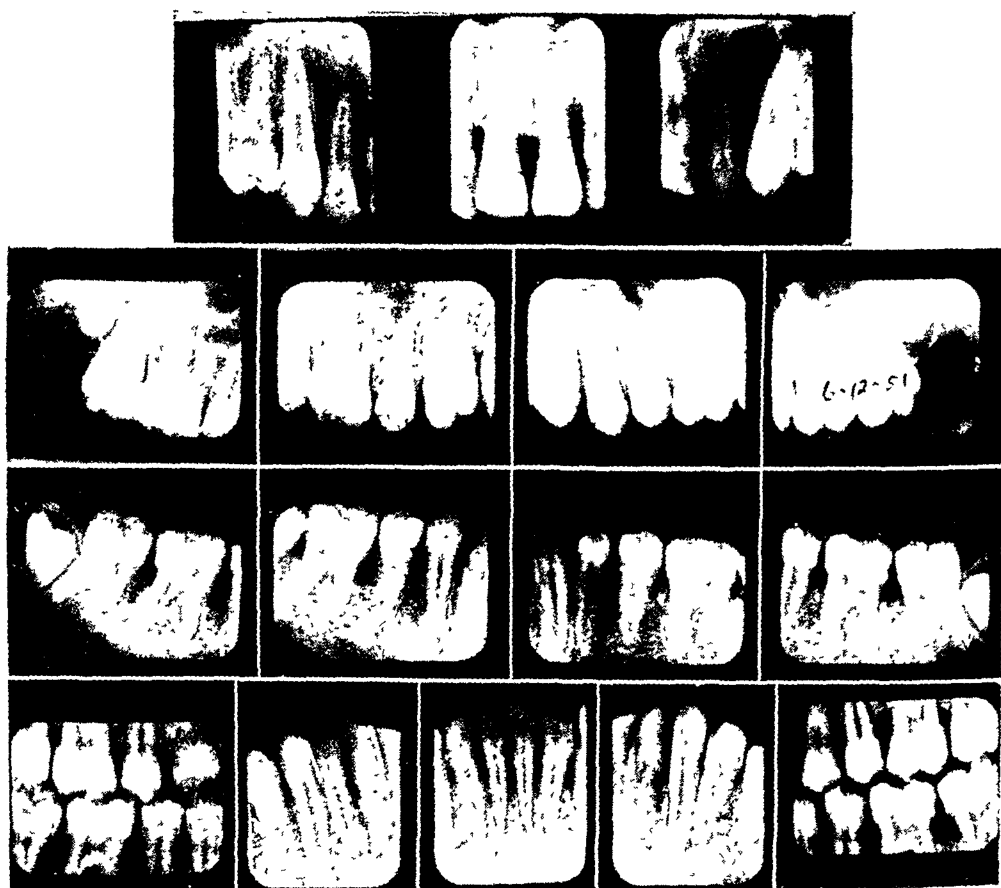


FIGURE 18 Radiograms of same case as FIGURE 17 Before systemic treatment

The radiograms were negative except for some probable epiphysitis of the theracolumbar spine. The patient mentioned that he had fleeting back pain in the lumbar spine upon heavy exertion.

The patient was placed on a low-calcium diet that ranged between 215 and 260 mg. of calcium per day. He was asymptomatic and afebrile throughout his entire hospital stay. Serum calcium values were high normal, and the serum phosphorus was increased. One 24-hour urine calcium excretion was normal; the other was slightly elevated. The patient was presented at Grand Rounds, where it was decided that, while the diagnosis of hyperparathyroidism was not substantiated by the evidence collected in the hospital, he should be put on an even lower calcium intake and studied further in the outpatient department. He was accordingly placed on a 150-mg. calcium diet, the lowest

vealed resorption of the alveolar and supporting bone about all of the teeth. Local therapeutic procedures by her dentist were unsuccessful. The patient was referred to an internist for a medical examination, and the following is a summary of the findings.

Childhood illnesses included 2 streptococcic sore throats and growing pains at the age of 7. There were no stigmata of rheumatic fever, however. The patient had had a sudden spurt of growth at the age of 11 and, at 11½, noted the onset of the menarche. Her menses were regular at 13 at intervals of 32 to 40 days, flowing 5 days, with no unusual features. At 14 to 16, she had a series of "colds" with periodic fever, that caused her to lose considerable time

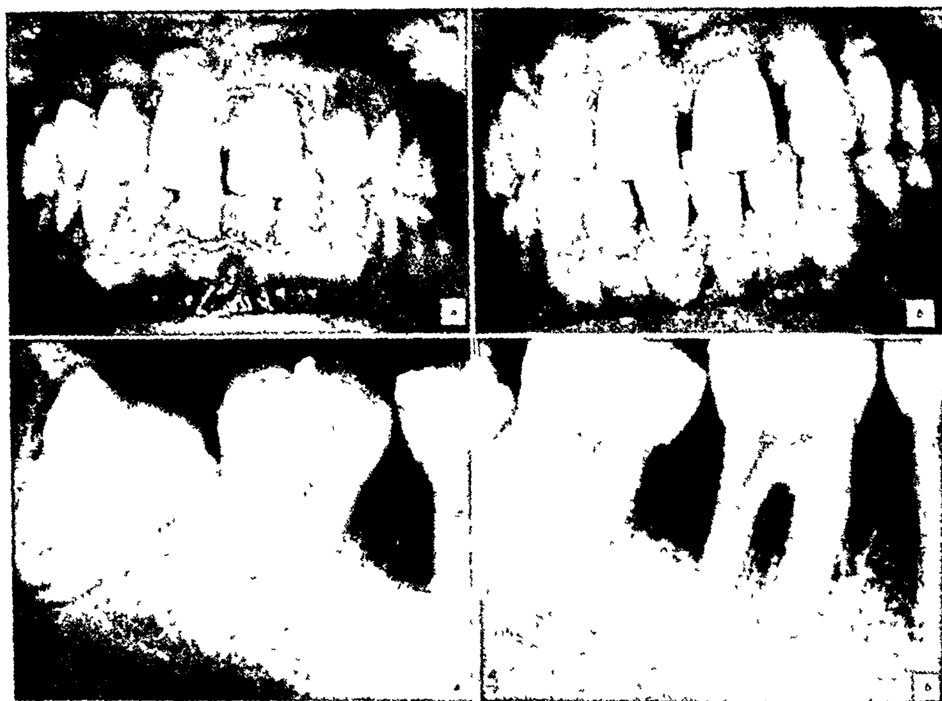


FIGURE 23 (*Top*). (*a*) Preoperative photograph. Marked gingival inflammation associated with local factors and an endocrine imbalance in 16-year-old girl (*b*) Postoperative photograph showing result achieved after regulation of systemic factor and local therapy.

FIGURE 24 (*Bottom*). (*a*) Preoperative radiogram of lower molar region in same case as FIGURE 23 (*b*) Postoperative radiogram showing healing of lesion between molars

from school. She is doing well at school, however, has made friends there, and is looking forward to going to college. Except for obesity, there has been no sign of abnormal thyroid function. Her B.M.R. is reported to have been low, and she has been on thyroid medication, 1 gr. daily. She weighs 155 lb. and has always gained and lost weight easily. In consequence her diet fluctuates widely, and she has taken regularly such vitamins as Mycebrine. She has been told that her blood pressure has been elevated.

Physical examination showed, in addition to the teeth and gingival gum findings, moderate obesity and a B.P. of 150/90. She was 5′7″ tall; the obesity was limited to the trunk. The oral temperature was 99.2° F. There were no skin keratoses, thickening, or striae. The tongue was normally papillated. The tourniquet test gave a negative reaction. There was no bone tenderness.



FIGURE 20 Radiograms of case seen in FIGURES 17 to 19 before and after treatment. Note healing of alveolar crest between molars in film on left

FIGURE 21 Radiograms of same case Before and after therapy Note healing of alveolar crest in film on left

FIGURE 22 Radiograms of same case Before and after therapy. Note narrowing of periodontal membrane space on film on left

correcting by moving bone calcium into the blood. I have not seen such a syndrome except in hyperparathyroidism, but it is a possibility. The high 17-KS excretion also is unexplained as is, unusual for this age, the hyperostosis frontalis. No definite evidence of adrenal abnormality other than this exists.

"To summarize, my feeling is that this young lady's troubles represent an altered glandular activity which may be functional and may spontaneously disappear as she matures.

"For the present, I believe a high-protein, low-calory (1500) diet, high vitamin, additional vitamin D, and the use of small doses of thyroxine and triiodothyronine are indicated. If the diarrhea persists, I would culture the stools and administer the vitamins parenterally. If there is no response, I believe further studies of her adrenal function and calcium metabolism are indicated."

This regimen proved successful in controlling this patient's systemic abnormality. The local intraoral factors then were removed, and healing was achieved (FIGURE 24).

These two histories, both local and systemic, have many similarities that make them companion cases. Of interest in this discussion are the unsuccessful results of mere local therapy, and the excellent oral response attained once the systemic factors had been eliminated.

The endocrine disturbances associated with uncontrolled diabetes have long been considered an important systemic factor in the causation of periodontal disease. Rapidly developing periodontal disease may be the clinical finding that first causes one to suspect uncontrolled diabetes, especially in the adolescent. Hirschfeld described fungating masses of granulatomatous tissue and multiple periodontal abscesses as characteristic gingival lesions in uncontrolled diabetes. Orban believes that the tissue changes found in the biopsy specimens of diabetics, especially vascular hyalinization and collagen fiber degeneration, could well explain the breakdown of the supporting tissues of the teeth. In examinations of the periodontium of diabetic patients many instances are seen of destructive changes that prove unresponsive to treatment. While it is difficult to evaluate properly the relationship of diabetes to periodontal manifestations, it may well be that the systemic background is a controlling or modifying factor in lesions initiated by local agents. It is recognized that diabetics have a lessened ability to combat infections; hence any role that nonspecific bacterial factors might play in the development of periodontal disease would be accentuated (Marble and Pollack, 1947). Other indirect and perhaps important factors in the development of periodontal disease in the uncontrolled diabetic are the depressed activity of vitamin C and the increased requirement of the vitamin B complex which is associated with the diabetic state (Burket, 1953); both being conditions that may have a detrimental effect on the tooth-supporting tissues. Diabetes should always be considered in cases of rapidly developing periodontal disease.

FIGURE 25 is an illustration of such a case. The patient, 54 years of age, complained of pain, fever, and general malaise. The teeth were sore at the touch; bleeding was easily induced. Dental examination disclosed several periodontal abscesses including those of the palatal aspect of the maxillary left central and lateral incisors and cuspid, and the buccal aspect of the man-

The thyroid was not palpable. The breasts were normal. The heart and lungs, and findings at rectal examination were all normal. The pubic hair was quite sparse. There were no abnormal reflexes.

A radiogram of the skull was made. The bones of the calvaria were average in width and architecture except for a slight hyperostosis frontalis interna. The base, including the sella, showed no abnormality. The pneumatic system was very extensive. There was swelling of the soft tissues at the roof and posterior wall of the nasopharynx, probably pharyngeal tonsils

The long bones of the right arm and right lower extremity showed no abnormality as to shape, architecture, or calcium. All epiphyses were united with the shafts. The wrists and hands showed complete development of the wrist bones and the fingers and their united epiphyses

The summary concluded that the changes in the frontal bone were unusual in a person of this age. Otherwise there were no abnormal findings (hyperostosis frontalis interna).

The laboratory findings were as follows.

Hemoglobin 13.8 gm., 89 per cent; red blood count, 4.5 million, white blood count, 10,000 Differential: polymorphonuclears, 58 per cent; lymphocytes, 35 per cent; large monocytes, 7 per cent, eosinophils, 0; basophils, 0, platelets, normal in number and shape

Urine reaction, acid, specific gravity, 1.020, albumin, negative; sugar, negative.

Sediment· W.B.C., 0 to 2, R.B.C., 0, casts, 0; others. mucus, squamous cells.

Sedimentation rate = 0.5 mm./min. corrected (high normal). Hematocrit 40 per cent. B.M R., 10 per cent, PBI, 7.0 γ per cent; I^{131} uptake, 30 per cent; special red cell test for triiodothyronine uptake on the low normal side.

Urinary 17-ketosteroids, 16 5 mg./24 hours (19.9 mg./24 hours creatinine-corrected), high for our laboratory, and test should be repeated.

Total protein, 7.3 gm./100 cc.; albumin, 4.2; globulin, 3.1. Alkaline phosphatase, 0.7; phosphorus, 4.2, calcium, 10.0 gm /100 cc.

Urine Sulkowitch ranged from 3+ (2 urines), trace to 1+ in urines sent to me.

FSH, 20 4 mU./24 hours.

Conclusion (by A Stone Freedberg*). "One cannot define a systemic condition responsible for her gum and teeth problem. There are, however, several findings which are unexplained and which may be related to this problem. The question whether an endocrine abnormality is responsible cannot be answered Endocrine abnormalities responsible for bone pathology usually produce generalized bone involvement. On the other hand, she is obese, has had had a low B M.R (the present B.M.R s are not basal since she had a cold), and the PBI is on the high normal side, all suggesting a lack of utilization of the thyroid hormone. We have described the response in such a syndrome to triiodothyronine. No evidence of hyperparathyroidism exists However, the calcium excretion on a low-calcium diet should be determined. The diarrhea and the positive Sulkowitch tests indicate a loss of calcium that she may be

* Beth Israel Hospital, Boston, Mass

dibular right second premolar. The remaining gingivae were inflamed, discolored, and retracted, and they bled easily; heavy calculus deposits were noted. Radiographically, there was marked loss of the marginal alveolus throughout. Because of the presence of multiple periodontal abscesses a detailed systemic history was taken. The patient described symptoms of excessive thirst, polyuria, and loss of weight. She was requested to have a physical examination. A diagnosis of diabetes was made. One month after institution of a proper diet and insulin she returned for periodontal therapy. The gingival changes may be observed in FIGURE 26. Therapy thus far had consisted of hot-water

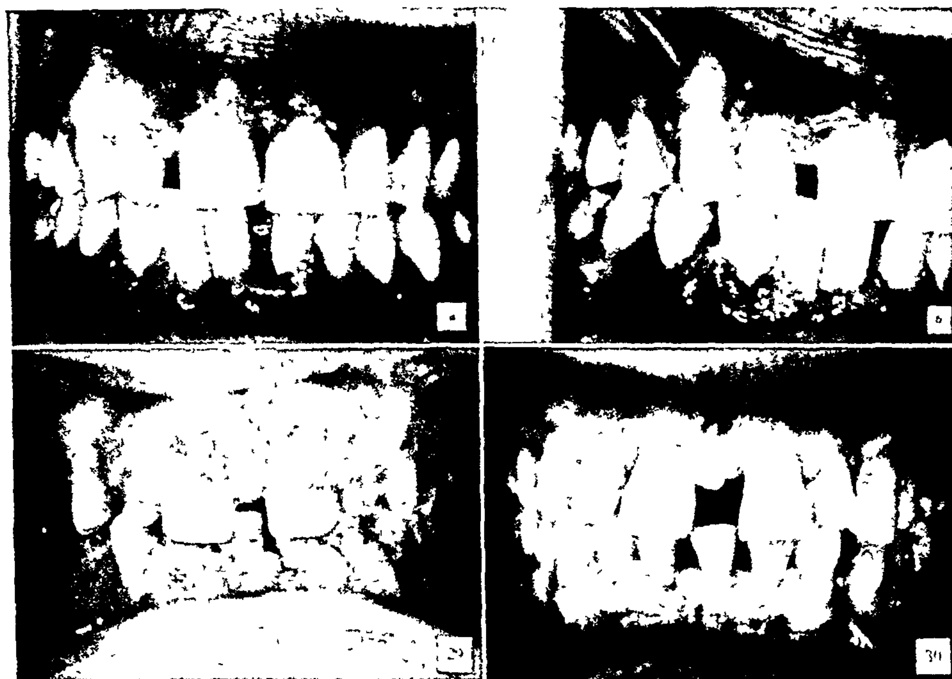


FIGURE 28 (*Top*, *a* and *b*). Mouth of an unrecognized diabetic after her dentist had performed gingivectomies throughout the mouth. Note gingival inflammation and deformities.
FIGURE 29. Marked gingival swelling and inflammation in an uncontrolled diabetic whose blood sugar level at time of photograph was 350 mg /100cc. Several periodontal abscesses were present.
FIGURE 30 Same case as illustrated in FIGURE 29. Note excellent result after scaling, subgingival curettage, and oral physiotherapy techniques, despite lack of control of diabetes.

rinses and brushing. Periodontal therapy was begun and the results may be seen in FIGURE 27.

Had periodontal therapy been instituted without adequate systemic regulation, many complications might have arisen. Thus symptomatic control of diabetes by means of a proper diet to ensure full nutritional rehabilitation and of insulin are necessary prerequisites for periodontal therapy. Control of the patient in this regard is the province of the physician.

In FIGURE 28 is shown a case in which the dentist proceeded with periodontal surgery without having taken an adequate history; it was a case of unrecognized diabetes. The overgrowth and marked gingival inflammation throughout the mouth demonstrate the inability of the tissues to heal because of the persisting endocrine imbalance.

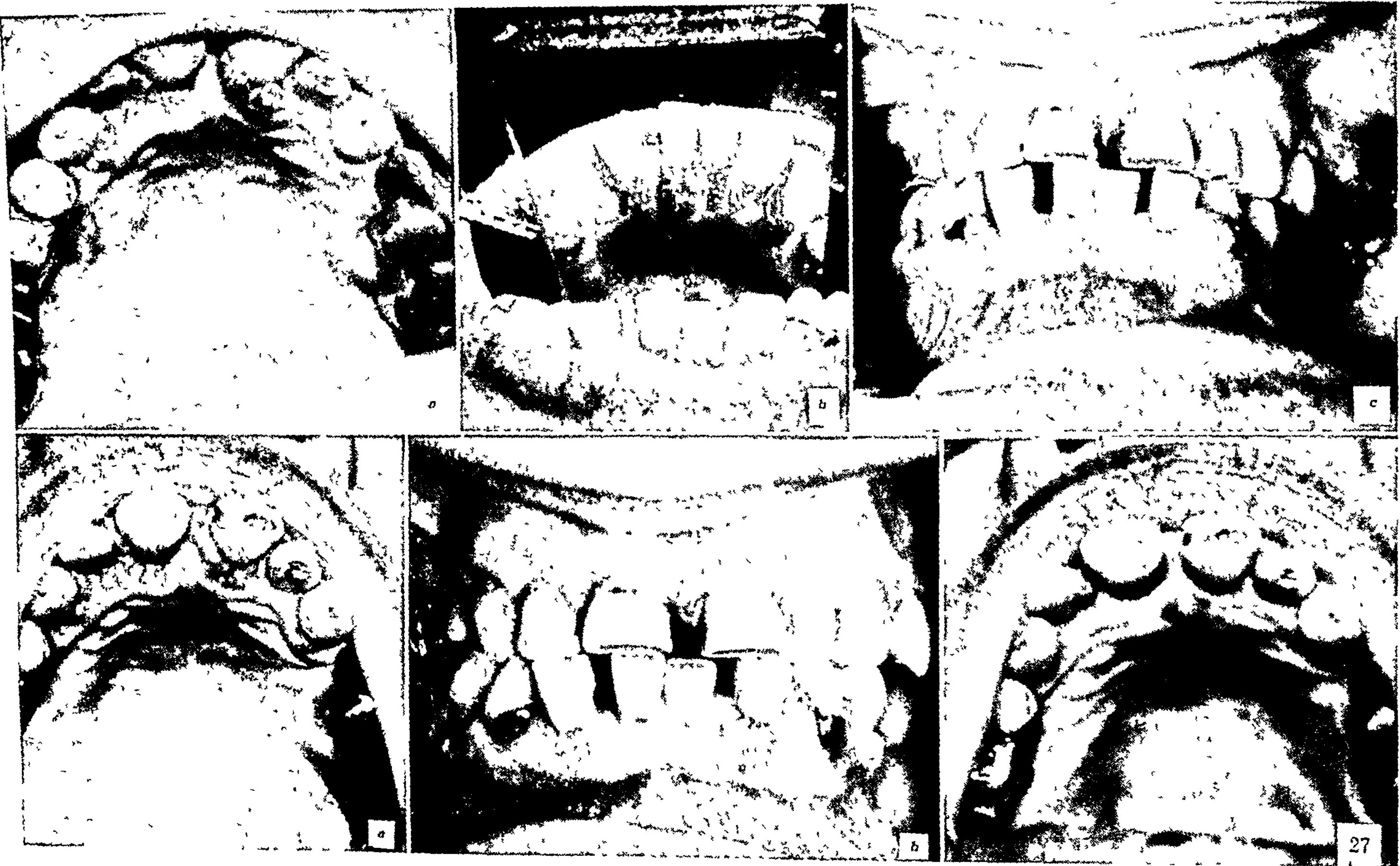


FIGURE 25 (*Top, a, b,* and *c*) Periodontal abscesses in a 54-year-old female with uncontrolled diabetes

FIGURE 26 (*Bottom, a* and *b*). Same case. Lesions had subsided with control of diabetic state Lesions still persisted because of local irritation

FIGURE 27. Same case Healing after periodontal therapy Here it may be seen that local and systemic factors contribute to the periodontal condition

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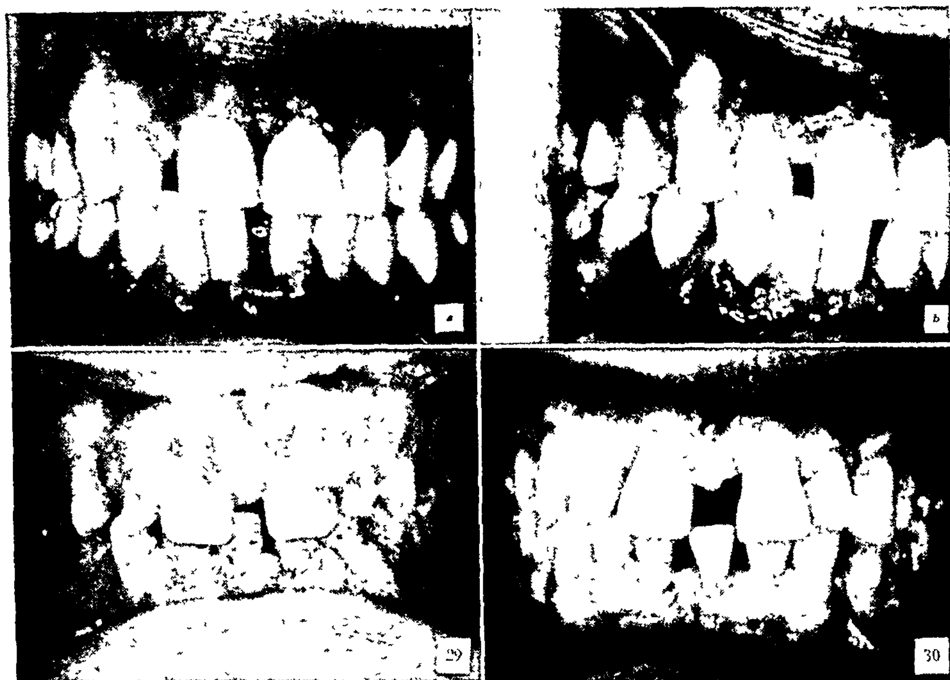


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FIGURE 29 shows an example of a recognized but poorly controlled diabetic. This patient was very lax and irregular in his insulin medication. There was very little attempt at dietary control, and his blood sugar level did not drop below 250 mg. per cent at any time during periodontal therapy. He was premedicated before and after each visit with antibiotics to reduce the danger of bacteremia from the local instrumentation. It is interesting to note the favorable response of the soft tissue in spite of the poor control (FIGURE 30). The radiograms indicate the severe resorption of the alveolar process (FIGURE 31).

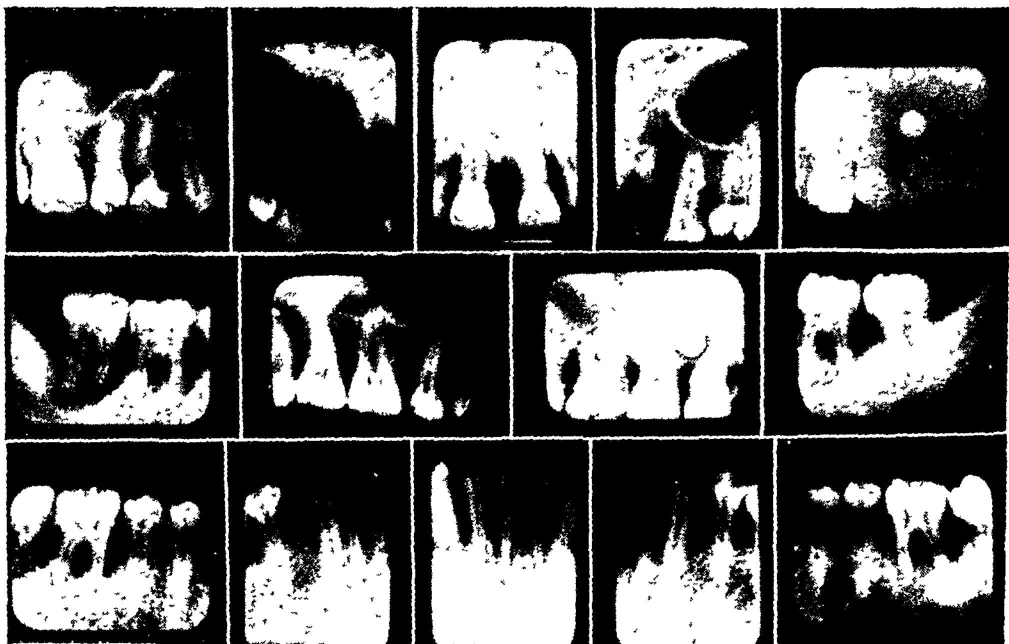


FIGURE 31 Radiograms of same case as illustrated in FIGURES 29 and 30. Note severe destruction of alveolar and supporting bone about many of the teeth

Nutritional Disorders

Burket (1952, 1953) points out that nutritional disorders, recognized or unrecognized, are without question the most common and, hence, perhaps the most important systemic diseases that may affect the periodontal tissues. The clinician should be concerned as much with the more frequent, mild, chronic nutritional disturbance as with severe acute deficiencies. Moreover, it should be stressed that, in addition to adequate food intake, good nutrition requires proper absorption and utilization of the food The latter phases of nutrition have been disregarded too frequently by the physician and dentist, and these factors, in the age range where periodontal disease is most prevalent are of great importance.

Much work has been done on the relationship between deficiency states and periodontal disease in animals (King, 1944, King and Glover, 1945; Stahl *et al*, 1955, 1957). The experiments have produced severe symptoms in the periodontium; while animal and human correlation (Burril, 1942) has yet to be proved, much insight into the relationship can be gained.

Some extremely interesting studies on subclinical scurvy have been reported

by Cheraskin *et al.* (1958), and by Karlson *et al.* (1959). Because of the rarity of classic scurvy these investigators have been collecting data on patients with no clinical evidence of vitamin deficiency but with laboratory proof of sub-

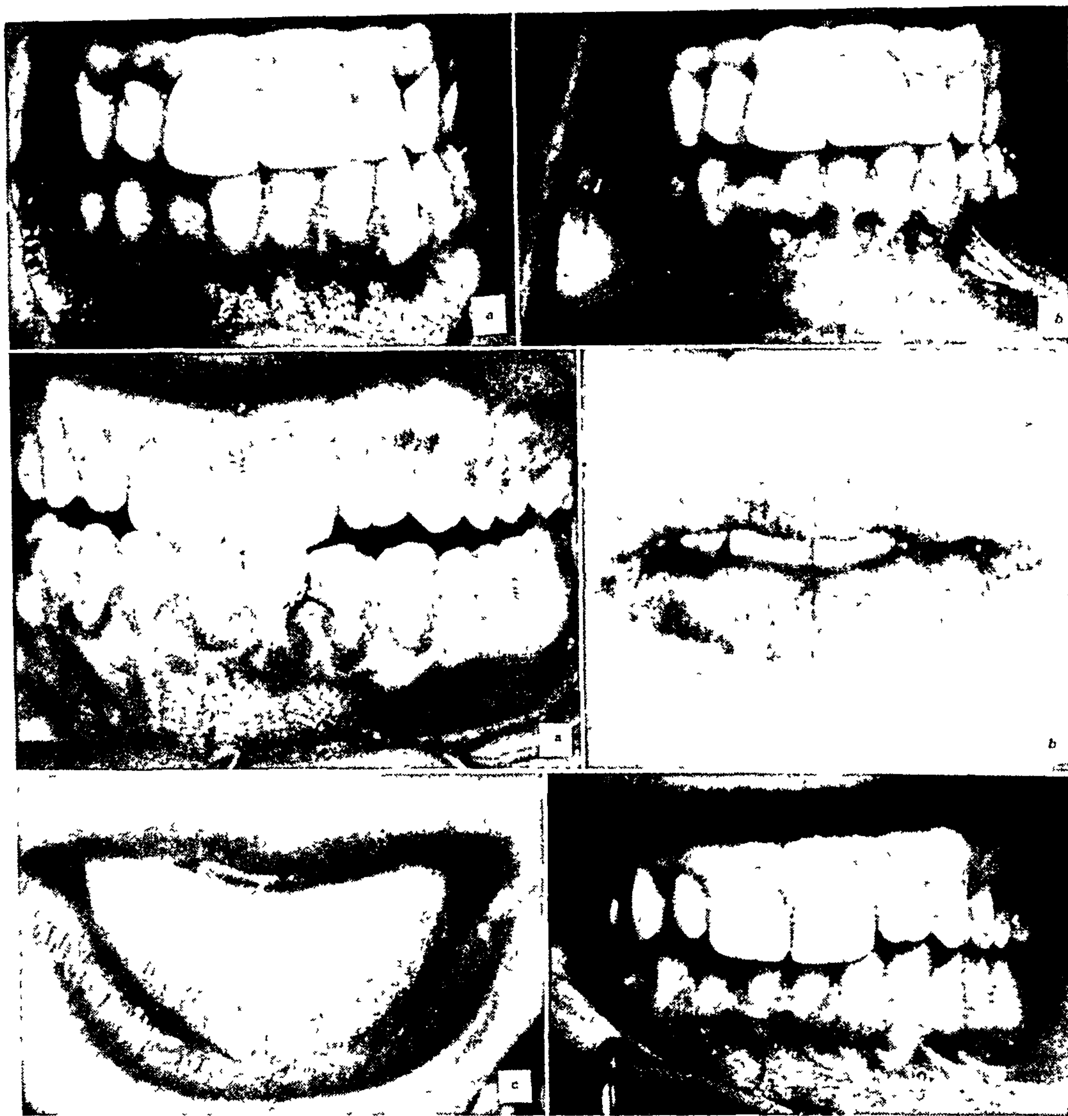


FIGURE 32 (*Top*) (*a*) Gingival inflammation and bleeding associated with local irritation and ascorbic acid deficiency (*b*) Healing following nutritional therapy and removal of local factors

FIGURE 33 (*Middle* and *bottom left*) (*a*) Gingival inflammation in a 16-year-old female with ulcerative colitis (*b*) Angular cheilosis associated with altered nutritional state in this patient. (*c*) Glossitis

FIGURE 34 (*Bottom right*) Same case as in FIGURE 33. Healing of gingival condition after nutritional rehabilitation and local therapy

clinical ascorbic deficiency. They correlated their observations with the patterns of tooth mobility. This is another demonstration of the multiplicity of disease-producing factors The classic studies of the scorbutic guinea pig by Follis (1943) again cite the dependence of hemarthroses of the limb upon local trauma as well as ascorbic acid deficiency.

FIGURE 32 shows inflammation and healing in a 19-year-old female who had

noted spontaneous gingival bleeding. She also complained of "spots in her eyes," and ophthalmologic examination revealed retinal hemorrhages. Calculus deposits were present on many of the teeth. The plasma-vitamin C level was zero, and the patient was put on a regimen of 1 gm. of ascorbic acid by mouth, citrus fruit, and polyvitamins every day. As the plasma-ascorbic acid level rose, the gingival bleeding decreased and the tissue tone returned. Local therapy was instituted to achieve a successful result.

FIGURE 33 shows a case of ulcerative colitis in a 16-year-old female. A glossitis and fissuring of the angles of the mouth developed along with the



FIGURE 35 (*Top*) (*a*) Exacerbation of gingival lesion after patient neglected her nutritional therapy. (*b*) Recurrence of angular cheilosis

FIGURE 36. Gingival inflammation in vitamin B complex deficiency state. Local etiologic factors were present.

FIGURE 37. Same case as in FIGURE 36 Reduction of inflammation after massive vitamin therapy No local therapy was performed.

gingival inflammation With adequate nutritional therapy the tongue and the cheilotic lesions improved; scaling and curettage were performed (FIGURE 34). The gingivae responded well, but after one year the patient became negligent with her diet and the oral lesions returned (FIGURE 35). After systemic and local therapy these lesions were eliminated. This may be an example of a combination of primary and secondary nutritional inadequacy. Primary deficiencies may be found in starvation, alcoholism, and medical or self-imposed dietary regimens. Secondary deficiencies may result from intestinal disease with inadequate absorption, from liver disease with defective metabolism, or from the increased demands of fever, hyperthyroidism, or pregnancy (Boyle, 1953).

FIGURE 36 illustrates a case in which several components of the vitamin B complex were lacking in a 22-year-old female. In addition to the typical

glossitis and angular cheilosis there were local irritations responsible for the gingival lesions. Local therapy of the gingival tissue failed to heal (FIGURE 37) until the nutritional status of the patient was regulated (FIGURE 38).

Other Systemic Alterations Affecting Oral Tissue Metabolism

The close relationship of physical health to the periodontium is illustrated in FIGURE 39, comprised of before-and-after photographs of a 31-year-old fe-

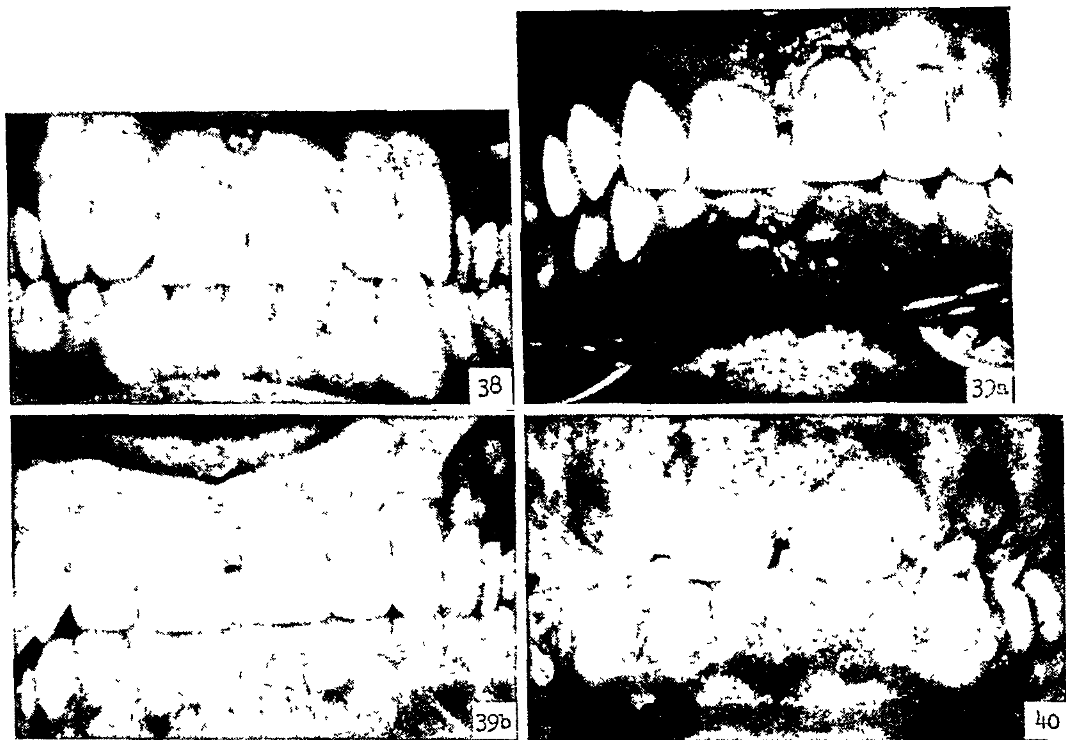


FIGURE 38 Same case as in FIGURES 36 and 37. Further reduction of gingival inflammation after local therapy of subgingival curettage

FIGURE 39 (*Top right* and *bottom left*). (*a*) Young woman aged 31 years, who had been a "blue baby" Note the discolored, boggy appearance of the gingivae The patient was operated on for the heart abnormality successfully, and one month later FIGURE 39*b* was taken Note the difference in tissue consistency and color. The case illustrates the dependence of gingivae on general blood supply and that systemic alteration also may affect the gingivae

FIGURE 40. Severe gingival hyperplasia in a 16-year-old female who was taking sodium Dilantin for 1½ years

male who was operated on for a congenital heart abnormality. The first picture shows the marked bluish color of the gingiva, and a slight inflammatory change. The second photograph was taken one month after the operation; note the change in the gingival color This case discloses the intimate relationship between the general blood supply and the gingiva

Sodium diphenylhydantoin (Dilantin)sodium, widely used in the treatment of epilepsy, is frequently associated with a marked gingival hyperplasia; gingival enlargement has been reported in from 6 to 50 per cent of patients receiving this form of sedation. The occurrence and degree of hyperplasia are not directly related to the dosage or duration of administration of the drug. Here again, local sources of irritation and poor oral hygiene are thought to be im-

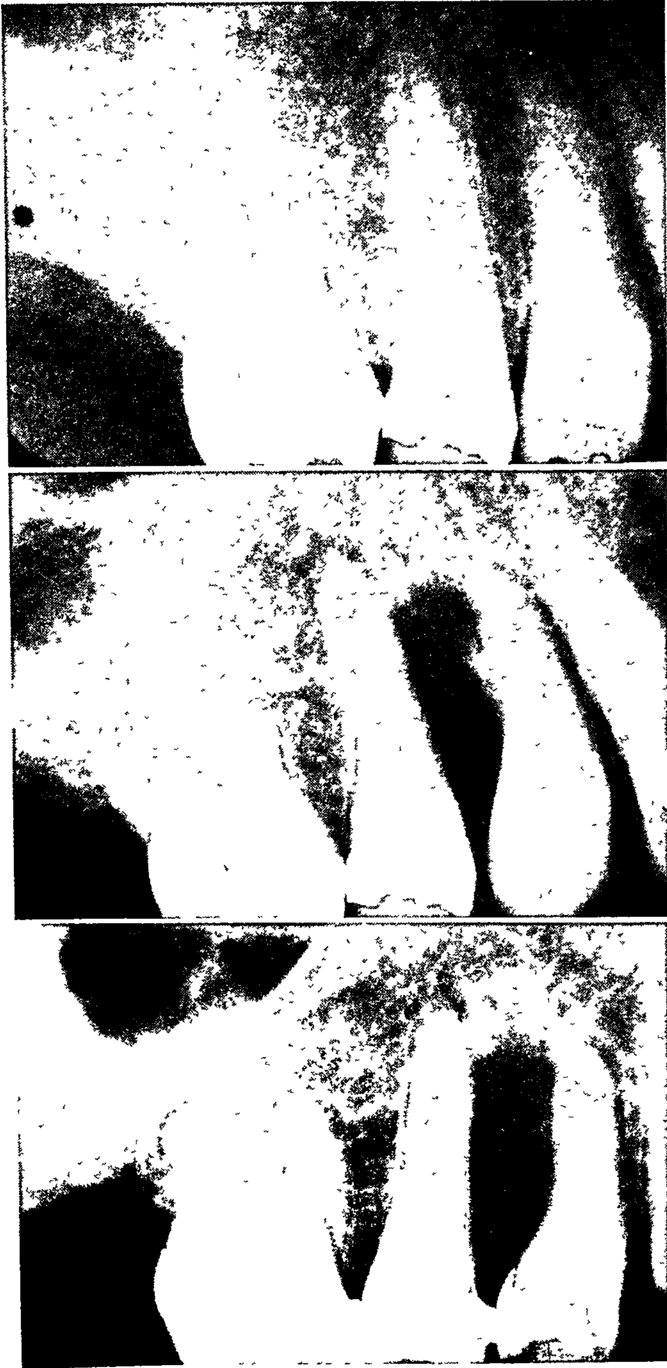


FIGURE 41. Young man with leukemia Upper radiogram taken 4 months before middle radiogram Patient developed leukemia during this period Local factor was food impaction between the 1st and 2nd bicuspids Lower radiogram taken 1 month later shows the joined restorations that eliminated food impaction. This is an example of the interaction between local factors and poor resistance because of a systemic alteration (Courtesy of Oral Diagnosis Clinic, University of Pennsylvania School of Dentistry.)

portant factors in the pathogenesis of this lesion (FIGURE 40). The hyperplasia does not seem to occur in edentulous areas. The physician should place the patient on another anticonvulsant before undertaking local therapeutic procedures. When the patient stays on Dilantin the proliferation tends to recur after local treatment.

The first clinical symptoms of acute leukemias, especially acute monocytic and myelogenous leukemia, may be noted in the mouth. They consist of a marked enlargement and, frequently, necrosis of the marginal gingival tissues and the cheek, and sometimes of the tongue. The earliest lesions usually are found in areas where there is some form of chronic irritation. FIGURE 41 shows radiograms of the maxillary premolar region in a young man with leukemia. An open space permitted food impaction between the two premolars.

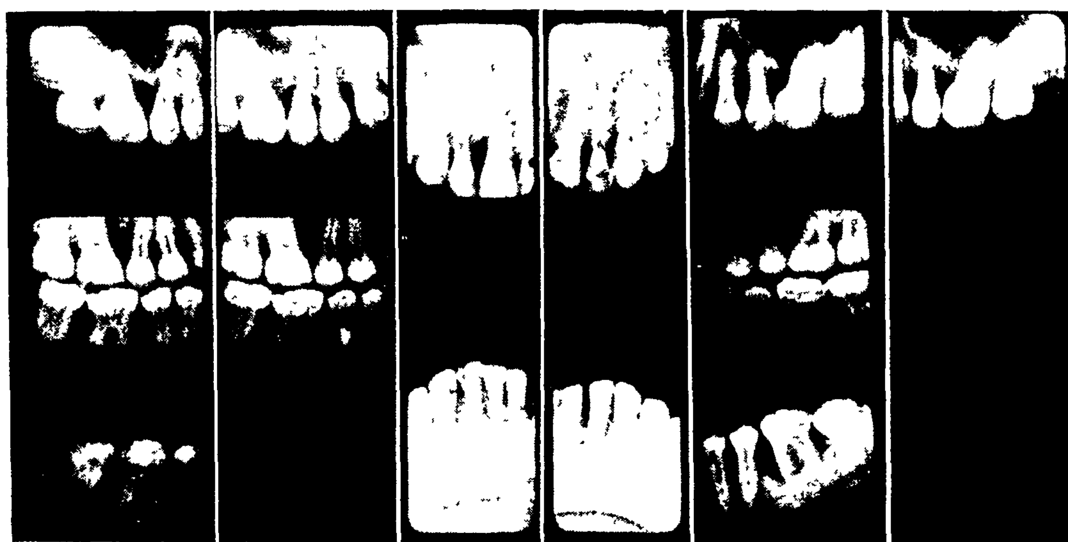


FIGURE 42. Full-mouth radiographs of a 16-year-old female. Note severe destruction in anterior and posterior regions (Courtesy of Samuel Stone.)

The second radiogram shows the severe destruction of alveolar process that took place in four months. The food impaction was corrected by a restoration, as seen in the third radiograph. The destruction was far more severe and rapid than one usually resulting from local factors alone

The Factor of Inheritance in Oral Disease Causation

In the above discussion of some of the important factors that favor the appearance and development of oral disease, we have been more concerned with their existence and mechanisms of action than with their origins. It has been intimated that many of these factors originate in interactions between the organism and its external environment. Even so, the organism must be so constituted as to be capable of acquiring the very characteristics that are consequent upon such interactions. As Forbus (1943) and others have pointed out, a constitution arises, insofar as is known, only through the process of inheritance, and so heredity must be regarded as an intrinsic factor in the pro-

duction of disease. Two roles of heredity may be recognized: (1) heredity may so condition an otherwise normally developed individual that increased susceptibility to injury arises upon contact with extrinsic agents of disease, and (2) it may so influence the embryonic development of the individual that the finished product is malformed In the latter case the developmental disturbance is in itself a disease that may result in death of the organism or may lead

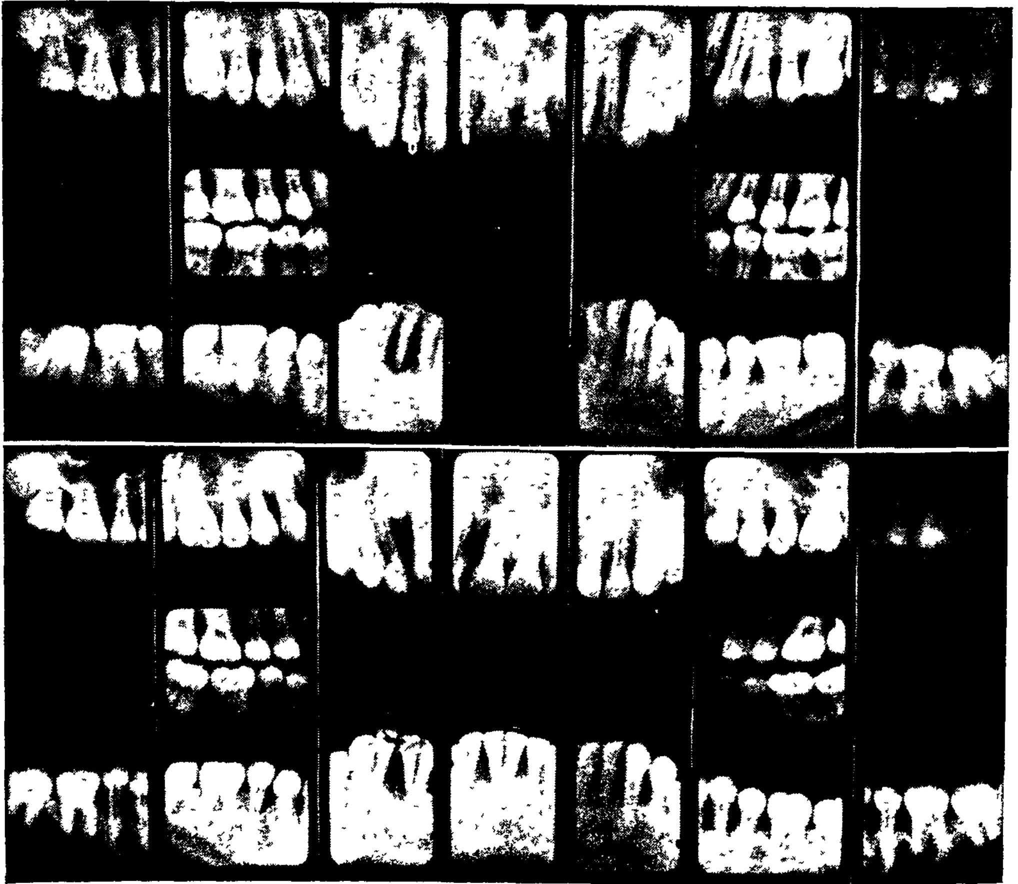


FIGURE 43 (*Top*) Full-mouth radiographs of an 18-year-old brother of patient of FIGURE 42 Destruction of osseous tissue is noted in anterior and posterior regions (Courtesy of Samuel Stone)

FIGURE 44 (*Bottom*) Full-mouth radiographs of a 20-year-old sister of patients illustrated in FIGURES 42 and 43 This sibling seems to exhibit the greatest damage in the periodontal structures (Courtesy of Samuel Stone.)

to other disease following the developmental period because of impaired adaptability. The sum of all the genetically determined, internal or intrinsic factors in disease determines what is often called individual, or group, constitution. To "constitution" in this sense must be attributed an enormous, if not the dominant, role in all production of disease

FIGURES 42, 43, and 44 are the intraoral radiograms of three siblings with periodontosis Both parents exhibited the typical periodontitis of the fourth decade of life. Unfortunately, complete laboratory and genetic studies were not possible in this case.

The possibility of an inherited weakness or inferiority of an organ must be considered in certain oral conditions. Reports of familial tendencies to periodontal disease are becoming more numerous in the literature. Although genetics is a highly advanced field of biology that approaches mathematical exactness and predictability, much further information is needed about inherited patterns that influence the metabolism or oral tissues (Forbus, 1943).

The field of genetics is one of the horizons of future research in the field of oral disease.

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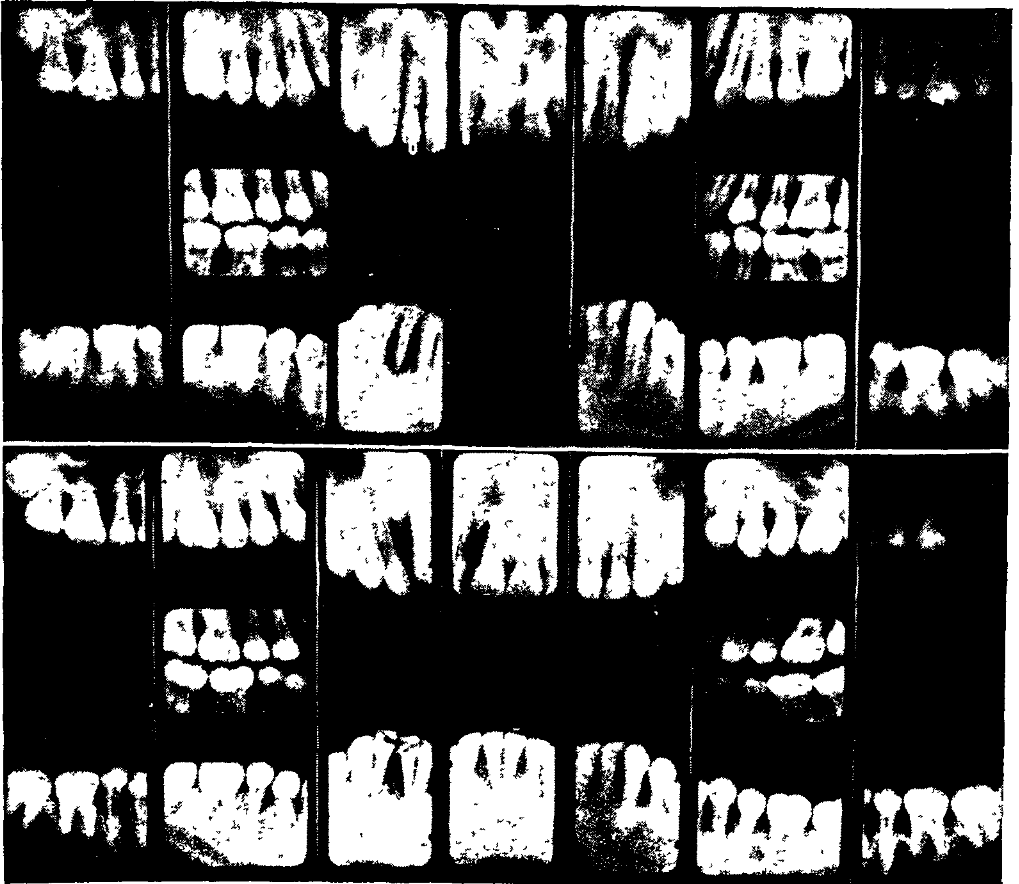


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FIGURES 42, 43, and 44 are the intraoral radiograms of three siblings with periodontosis. Both parents exhibited the typical periodontitis of the fourth decade of life. Unfortunately, complete laboratory and genetic studies were not possible in this case.

animals are placed on experimental diets at 16 days of age, immediately after weaning.[5] We were then ready for the next phase of the study, in which 2 groups of animals, maintained for 30 days on high- and low-phosphate diets, respectively, were subsequently given high sucrose cariogenic diets for from 12 to 14 weeks. The calcium and phosphate contents of the cariogenic diets were maintained at the same levels as in the postweaning diets. We presented our preliminary findings on caries susceptibility before the Josiah Macy, Jr., Conference on Metabolic Interrelations held in New York, N. Y., in 1952[6] (TABLE 1). The high-phosphate diet was found to be decisively cariostatic. Both the extent and number of carious lesions were reduced to one fourth in the high-phosphate group. Phosphate was added to the diet in the form of

TABLE 1*
INFLUENCE OF DIET ON CARIES SUSCEPTIBILITY

Diet	Number of animals	Number of carious lesions (mean values)	Extent of carious lesions (mean values)
High Ca, Low PO_4	41	10.8	22 5
Low Ca, High PO_4	27	2 7	5 6

* Reproduced with permission from the *Transactions of the Josiah Macy, Jr, Conference on Metabolic Interrelations* [6]

TABLE 2
CARIOSTATIC EFFECT OF HIGH-PHOSPHATE DIET

Authors	Date	Experimental animal	Cariogenic diet
Sobel[6]	1952	Cotton rat	High sucrose diet
Wynn *et al* [8]	1956	Albino rat (desalivated)	High sucrose diet
Strålfors[11]	1956	Hamster	Cereal diet
McClure[9]	1958	Albino rat	Cereal diet
Nizel *et al* [10]	1958	Hamster	Keyes diet

the soluble disodium hydrogen phosphate in amounts such that the phosphate content of the diet was increased to 0 71 to 0.74 per cent. The cariostatic effect of dietary phosphate was subsequently confirmed and extended in our own laboratory[7] and in a number of other laboratories where different animals and different methods of caries production were utilized[8-12] (TABLE 2) Wynn *et al.*,[8] working with desalivated albino rats, kept them on a sucrose diet; McClure,[9] using the same species, eliminated the need for desalivation and used a lysine-deficient cereal diet for caries production. Nizel *et al.*[10] and Strålfors[11] worked with hamsters and produced caries with a cereal diet. Preliminary studies by Strålfors (unpublished data) indicate that dietary phosphate supplement is effective as a cariostatic agent in humans. Although the above studies[7-11] utilized different animal species and different cariogenic diets, the over-all results are in agreement with regard to the cariostatic effect of soluble

Part II. Tooth Tissues

INTERRELATIONSHIP OF TOOTH COMPOSITION, BODY FLUIDS, DIET, AND CARIES SUSCEPTIBILITY*

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Introduction

For many years this laboratory has been concerned with the composition of mineralized tissues and the relationships that exist between the composition of the mineral fraction of the tissue, the composition of the body fluids, and the composition of the diet This paper will emphasize those portions of the investigations that have bearing on the caries problem. As will be developed later, some of the relationships have been investigated extensively, while others require further study.

Cariostatic Effect of Phosphate

In 1942, in a paper[1] presented before the American Chemical Society, Buffalo, N Y., my colleagues and I reported a relationship between the mineral composition of bone and the composition of blood serum and diet When animals were fed a high-phosphate diet, bone carbonate, which is more soluble than bone phosphate, was low, while a low-phosphate regimen produced bones relatively high in carbonate content At that time it was postulated that, if such relationships also govern tooth composition, caries susceptibility is affected In particular, it was expected that the tooth with more soluble mineral content would be more vulnerable to the caries process, while the tooth whose mineral is less soluble would be caries-resistant Thus, diets containing a high percentage of soluble phosphate were expected to be cariostatic.

By 1948 we completed studies in which it was demonstrated that the mineral composition of the incisors of the Wistar rat can be influenced by the diet and reaffirmed that dietary composition is related to blood composition.[2] The following year, through the mediation of C G King of the Nutrition Foundation, New York, N Y , and R R Williams of Columbia University, New York, N. Y , a team was organized consisting of James H. Shaw of the Harvard School of Dental Medicine, Boston, Mass , and Albert Hanok and myself of the Jewish Hospital of Brooklyn, to test these concepts in the cotton rat. This rodent, which is phylogenetically not a true rat, was chosen for our studies because of its high susceptibility to caries when maintained on a high-sugar diet.[3,4] The first question investigated was whether the composition of the molars can be modified under the influence of the diet, as was the case for the continually growing incisors. By 1951 we were able to demonstrate that significant differences in the composition of molars of the cotton rat can be produced when the

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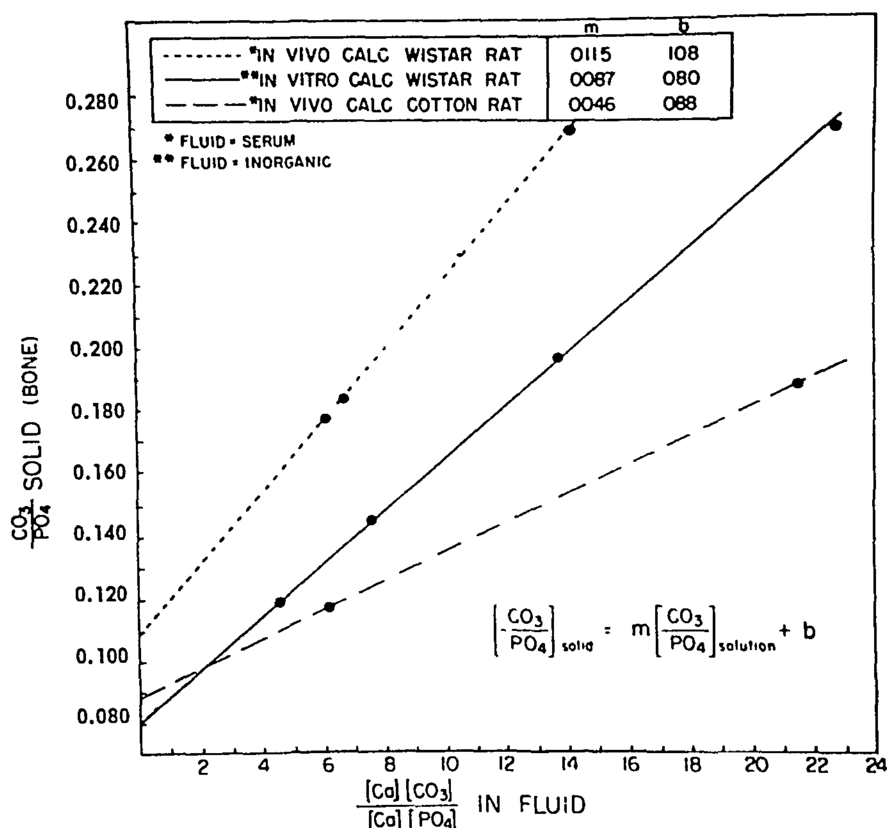


FIGURE 1. $CO_3:PO_4$ ratios of bone mineral calcified *in vivo* and *in vitro* in relation to fluid composition.

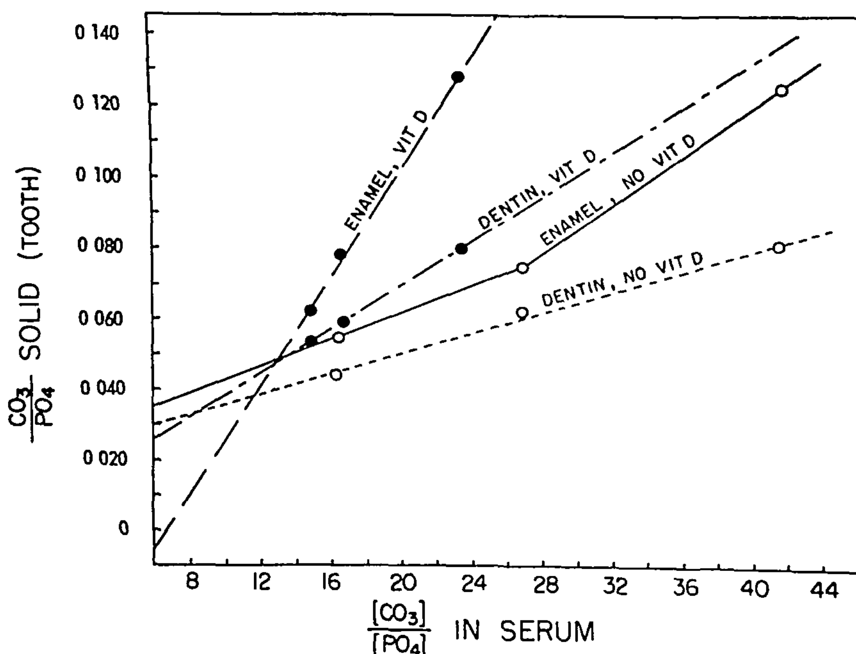
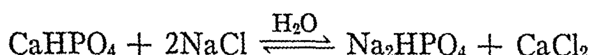


FIGURE 2. $CO_3:PO_4$ ratios of tooth mineral in relation to serum composition in the incisors of the Wistar rat after 45 days on experimental diets

phosphate. McClure and Muller[12] pointed out that the cariostatic effect of $CaHPO_4$, which is relatively insoluble, is enhanced markedly in the presence of NaCl, and he attributed the effect to the solubilizing action of NaCl. It must be borne in mind that, when phosphate is present in the diet in the form of calcium acid phosphate, it is converted in the presence of sodium chloride to the more soluble disodium hydrogen phosphate



$$CaHPO_4 + 2NaCl \xrightleftharpoons{H_2O} Na_2HPO_4 + CaCl_2$$

Interrelation of Diet, Blood, Bone, and Tooth Composition

The relation between composition of diet and of blood serum is given in TABLE 3. As the dietary phosphate is increased, the serum phosphate rises.

TABLE 3
RELATIONSHIP OF THE SERUM CALCIUM AND INORGANIC PHOSPHORUS LEVELS TO THE CALCIUM, PHOSPHORUS, AND VITAMIN D CONTENT OF THE DIET IN RATS

Diet		Serum*					
		Without vitamin D			With vitamin D†		
Ca (%)	P (%)	Ca (mg %)	P (mg %)	CO_3:PO_4 (mole ratios)	Ca (mg. %)	P (mg %)	CO_3:PO_4 (mole ratio)
A. Experimental period, 30 days							
1.20	0 121	11 7	2 1	40.0	13 3	3 4	25.7
0 20	0 124	9 4	4 7	17 6	11 1	6.0	13 2
0 03	0.759	5 6	7.5	9.9	8.8	8.4	9.3
B. Experimental period, 45 days							
1 20	0 121	12 3	1 8	41 7	15 0	3.4	22.2
0.20	0.124	10.0	2.6	27 1	11 2	4.6	16 7
0.03	0.759	5.1	4 9	16 1	8.9	5.2	14 9

Two groups of Wistar rats 23 days old were placed on the experimental diets for 30 and 45 days, respectively Each of 6 litters was divided among the 6 groups in each experiment
* Mean values for serum.
† One hundred I.U of vitamin D, daily

The change is not so striking in animals fed vitamin D, because this metabolite tends to elevate both calcium and phosphate in blood serum, particularly the member of the pair that is low.[13] Since serum carbonate, under these conditions, is essentially constant,[2,7] serum CO_3:PO_4 ratios are dependent on the phosphate content of the diet. A direct relationship between CO_3:PO_4 ratios of the fluid and of bone composition is shown in FIGURE 1. In calcification *in vitro* the fluid was an inorganic calcifying medium, while in calcification *in vivo* the fluid CO_3:PO_4 ratios were obtained from blood serum. A similar relationship between fluid and incisor composition in the Wistar rat is illustrated in FIGURE 2.[2,7,14] It is worth pointing out that vitamin D changes the type of relationship between serum and tooth mineral composition. FIGURE 3 shows

Caries Hypothesis

The caries hypothesis that was formulated depended on the relationships established between the compositions of diet and body fluids, which in turn regulate the composition of growing teeth. We postulated that caries susceptibility would be increased on a low-phosphate diet, while a high-phosphate diet

TABLE 4
CARIES SUSCEPTIBILITY AND HIGH PO_4 DIET IN THE COTTON RAT[17]*

Diet	Period	No of animals	Number of carious lesions‡ (mean values)	Extent of carious lesions‡ (mean values)
High Ca, Low P, 4 weeks; + sucrose 12 to 14 wks	1949–1952 1953–1954	61 30	11 0 15.6	25.2 44.9
Low Ca, High P,† 4 weeks; + sucrose 12 to 14 wks.	1949–1952 1953–1954	41 38	5.3 8 6	13.1 22.9
p	1949–1952 1953–1954	— —	≪0 01 <0 005	<0.01 <0 005

* Age of animals at beginning of experiment was 16 days Litters divided between the 2 experimental diets

† Phosphate added as 3 2 to 3.5 per cent Na_2HPO_4

‡ Caries evaluated by James H Shaw, Harvard School of Dental Medicine, Boston, Mass

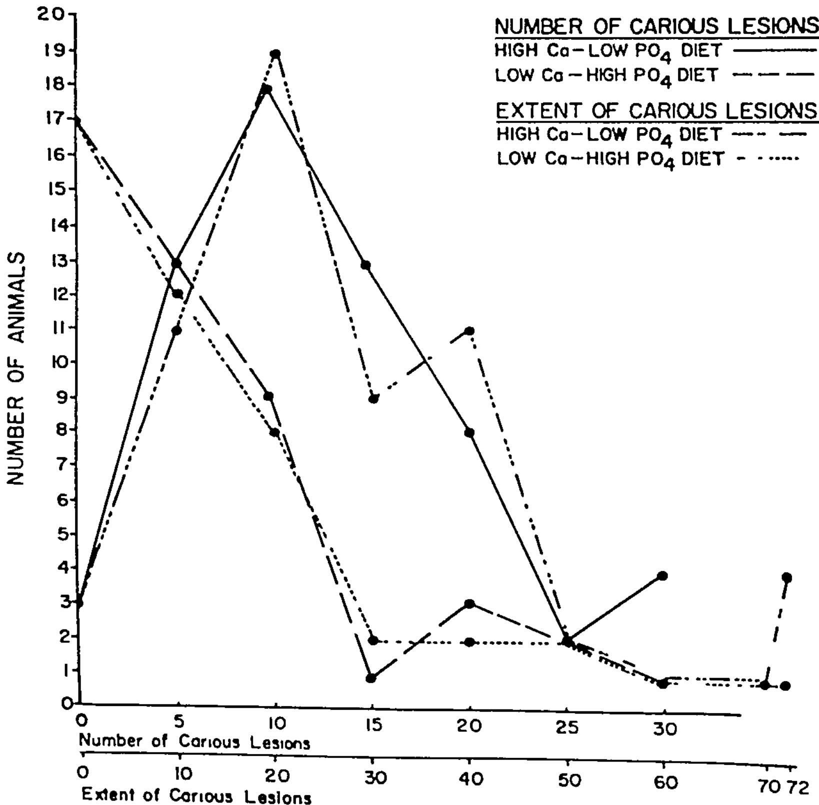


FIGURE 4 Distribution of number and extent of carious lesions during the experimental period 1949 to 1952

that, in the cotton rat, serum composition influences the CO_3:PO_4 ratio of not only the femur and of the enamel and dentin of the upper incisors, but also of the enamel and dentin of the upper molars. It must be noted that the extrapolations to zero (FIGURES 1, 2, and 3) are not valid, although the relationships are linear in the observed range.

Caries Susceptibility and High-Phosphate Diet in the Cotton Rat[15]

The results of the extended studies on caries susceptibility with high- and low-phosphate diets are shown in TABLE 4. During the period from 1949 to

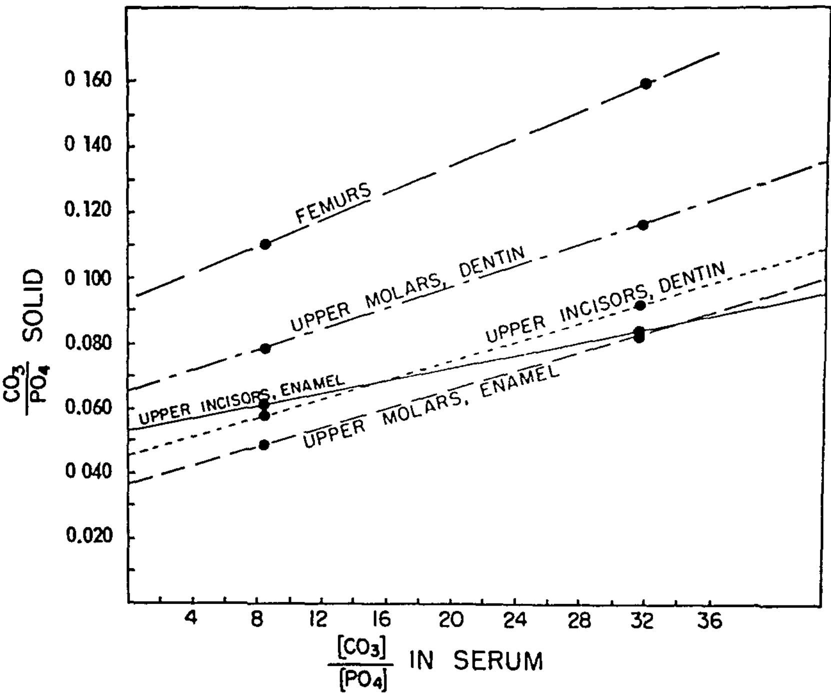


FIGURE 3 CO_3:PO_4 ratios of femur and enamel and dentin of molars in relation to serum CO_3:PO_4 ratios in the 16-day-old cotton rat on an experimental diet for 30 days.

1952 relatively wild rats were utilized During the second experimental period, 1953 and 1954, the animals were tamed by constant handling and inbreeding in our laboratories. Each litter was divided and put on 2 separate experimental diets. Between 1949 and 1952, the rats on the low-phosphate diet averaged 11 lesions, with an extent of 25.2, whereas the rats fed the high-phosphate diet averaged 5.3 lesions, with an extent of 13 1. Parallel results were obtained in the second experimental period, 1953 and 1954: rats on the low-phosphate diets averaged 15.6 lesions, with an extent of 44.9, and rats on the high-phosphate diets averaged 8.6 lesions, with an extent of 22.9. FIGURE 4 illustrates the distribution of number and extent of carious lesions for the first period; a comparable distribution was observed in the later period. It is evident from these studies that the number and extent of carious lesions were significantly lower in animals on the high-phosphate diet (all p values were less than 0.01).

enamel, each mole of phosphate going into solution was accompanied by as much as 20 moles of carbonate, and suggested that this caused breakdown of the crystal lattice. His general premise that carbonate may be the Achilles' heel of caries is compatible with our view. However, in view of the present concept that carbonate is on the surface rather than in the lattice of the tooth crystal,[16,26,28] his suggestion that removal of carbonate results in breakdown of the crystal lattice is probably not valid.

If the carbonate is adsorbed on the crystal surface, then volume changes following loss of carbonate would be greater in the high-carbonate than in the low-carbonate tooth crystal. An index of this volume change may be obtained

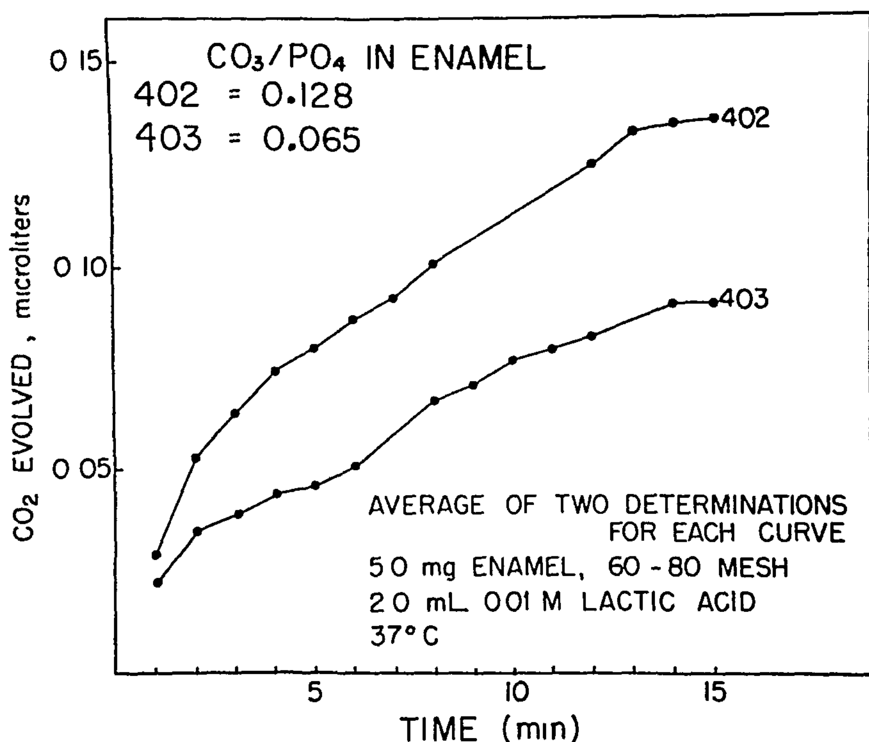


FIGURE 6. Evolution of carbon dioxide in acid-treated enamel in relation to the CO_3:PO ratio of the enamel.

by comparing the CO_3:PO_4 molar ratios obtained after feeding high calcium-low phosphate and low calcium-high phosphate diets to an extensive series of cotton rats.[14] The mean CO_3:PO_4 ratio for the enamel in the upper molars was 0.131 on the low-phosphate diet, as compared to 0.076 on the high-phosphate diet. Our calculations show that the carbonate portion of the adsorption layer, expressed as calcium carbonate, represented 7.9 and 4.6 per cent, respectively, of the volume occupied by the hydroxyapatite portion of the enamel crystal.[29] Moreover, the uneven distribution of carbonate in enamel found by Little and Brudevold[30] suggests that there are areas in the high-carbonate tooth where this difference is even more pronounced. Since we are dealing with a distribution of carbonate, it may be of interest to consider the maximum theoretical carbonate that may be bound to an apatite crystal surface. Carlström[31] calculated that the unit cells at the surface of the (10·0)-face can vary in

would decrease caries susceptibility. The early version of the working hypothesis[1,2] was as follows:

(1) Acids dissolve carbonate preferentially from bone and tooth mineral.[16-20] The more rapid dissolution of carbonate from high-carbonate dentin and enamel is illustrated in FIGURES 5 and 6.

(2) $Ca_3(PO_4)_2$ is more soluble in the presence of carbonate, due to the formation of un-ionized carbonate-phosphate complexes[21,22]

This hypothesis, in its broader sense, applies to any factors in the tooth composition that affect its solubility. For example, in inorganic model systems,

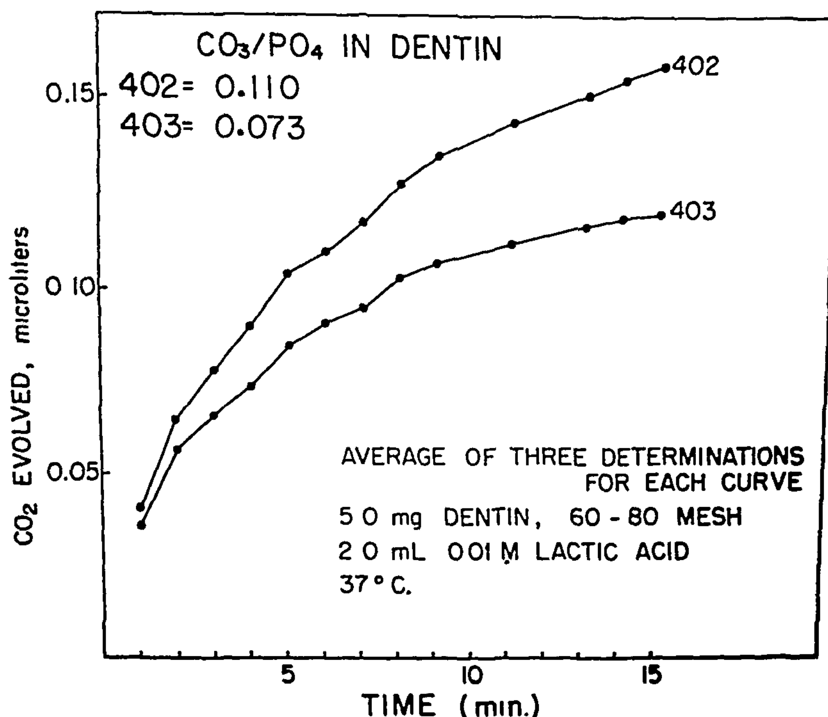


FIGURE 5. Evolution of carbon dioxide in acid-treated dentin in relation to the CO_3:PO_4 ratio of the dentin

hydroxyapatite-containing citrate would have increased solubility;[23] increased carbonate content increases the solubility of hydroxyapatite; fluoride, on the other hand, decreases the solubility of hydroxyapatite. As seen in FIGURE 7, a schematic diagram of the present concept of bone and tooth mineral crystals, most variable components are contained in the adsorption layer. Owing to the extremely small size of the crystal, this layer occupies a considerable proportion of the total volume. Assuming a relatively soluble adsorption layer, one can visualize the preferential dissolution of adsorbed carbonate initiating the caries process.

It has been demonstrated for bone and tooth as well as for carbonate-containing apatite minerals that carbonate is preferentially dissolved by dilute acids[16-19,25] and is preferentially lost from enamel in early clinical caries[27] Hardwick[20] showed that, in the initial stage of acid dissolution of powdered

that the inorganic portion of tooth is attacked first in caries, but would be inconsistent with an hypothesis wherein initiation is postulated in the organic matrix.

The uneven distribution of carbonate in enamel[30] suggests that gross analysis may mask the presence of localized high concentrations of carbonate leading to caries susceptibility. This may be a factor in the results obtained by Wynn *et al.*,[8] who found a relationship between dietary phosphate and caries in agreement with our study, yet were unable to detect differences in the composition of the total enamel tissue. In addition to the use of a different species of rodent, the much more fully developed teeth of their starting animals (26 days of age) as compared to ours (16 days of age) would be expected to show less influence of diet on composition upon analysis of the total enamel. However, marked changes in composition may have occurred in the surface layer of enamel. Moreover, this surface enamel is in more dynamic equilibrium with the diet and the ever-present oral fluids than the deeper layers of the enamel.

Diet and caries susceptibility have been correlated in several studies that have placed emphasis on the Ca:P ratio without regard for the absolute concentrations of calcium and phosphate.[8,9] It has been demonstrated, however, by Shohl *et al.*[32,33] and by Kramer and Howland[34] that the influence of dietary calcium and phosphate depends not only on the Ca:P ratios, but also on the absolute level of calcium and phosphate. For example, at a fixed ratio of Ca:P, the serum phosphate and the percentage of bone ash increases with the increase in absolute level of the salts, while the degree of rickets decreases. A diet with a Ca:P ratio of 2.39 at a phosphorus level of 0.33 per cent caused rickets, whereas a 2.23 ratio with the phosphorus increased to 0.51 per cent made for a nearly normal ash content.[35] Moreover, the presence of other dietary factors, notably vitamin D, will also profoundly influence the effects of a particular Ca:P dietary ratio.[6,36] It is inadequate to describe a diet with respect to its Ca:P ratio unless cognizance is taken of the influence of the absolute levels of calcium and phosphorus and of additional dietary factors. Serum levels of calcium, phosphorus, and carbonate are of value in assessing the effect of dietary concentration of these minerals, since they are related to tooth and bone composition.[2,6,36,37]

It is likely that many dietary factors can affect organic[38,39] and inorganic[40] composition, and thereby caries susceptibility. For example, subtle *in vitro* changes in the structure of the dentinal macromolecules modify the response of dentinal tissue to bacterial collagenase, either increasing or decreasing its resistance to attack.[41] The role of many trace minerals,[42] while correlated with caries susceptibility, is poorly understood. A careful study of the composition and structural changes of both inorganic and organic portions of the tooth in relation to caries susceptibility is indicated. Moreover, this would clarify the influence of factors in the oral environment.

Effect of Oral Fluids

Bone composition is completely under the influence of internal body fluids and "local factors." While this is true of teeth prior to eruption, after eruption the oral environment may also affect composition. The experiments of

composition from $Ca_6(PO_4)_6(OH)_2^{-8}$ to $Ca_{14}(PO_4)_6(OH)_2^{+8}$. In the latter case, if 4 carbonate ions are surface-bound to satisfy the valence requirements, then carbonate dissolution would cause an appreciable change in the volume of the

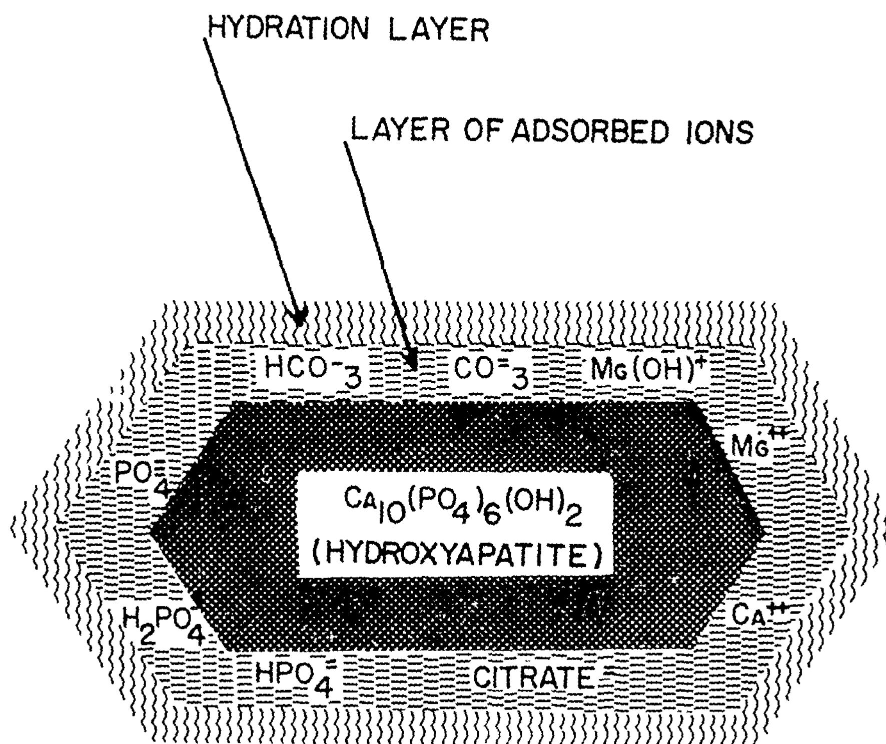


FIGURE 7 Schematic diagram of bone crystal in body fluid The proportions of the layer of adsorbed ions and the hydration shell shown are approximate. The carbonate portion of the adsorption layer would occupy from 3 to 16 per cent of the volume occupied by the hydroxyapatite crystal This volume was calculated by assuming that the specific gravities of calcium carbonate ($CaCO_3$) and hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ are about the same, in which case 10 carbonate ions as calcium carbonate and 6 phosphate ions as hydroxyapatite occupy equal volumes By multiplying the CO_3:PO_4 ratio (calculated from Table 1 of Sobel, 1955[7]) by 0.60 (6:10), the relative volume occupied by the surface carbonate compared to the apatite crystal volume is obtained A second assumption is that 1 mole of adsorbed calcium carbonate occupies the same volume as it would if it were an independent solid.

The actual volume occupied by the layer of adsorbed ions is probably greater than the calculated value, since this layer contains other ions such as citrate, $HPO_4^{=}$, and $Mg(OH)^+$ In addition, the specific gravity of hydroxyapatite is slightly higher than that of calcium carbonate

There is a tightly bound "hydration shell" surrounding and within the adsorption layer, containing 18 mM of water per 1 gm hydroxyapatite, according to Myers' recent report [24] This would be approximately 5 4 per cent of the volume occupied by the hydroxyapatite crystal

crystals, where almost one half of the unit cells are at the surface. At these focal points, where there is a build-up of the carbonate concentration, according to the law of mass action there would be increased sensitivity to acid attack, followed by removal of carbonate. The resultant decrease in crystal volume creates a crevice between the remaining crystal and the organic matrix. It would follow from the above considerations that high-carbonate teeth should be more susceptible to caries This discussion is consistent with the concept

that the inorganic portion of tooth is attacked first in caries, but would be inconsistent with an hypothesis wherein initiation is postulated in the organic matrix.

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Sognnaes *et al.* suggest"... a liquid-solid relationship between saliva and enamel similar in kind, if not in magnitude, to that existing between extracellular connective tissue and the mesenchymal hard tissues."[43] The oral environment particularly affects the outermost surface of the tooth, but there is also evidence for penetration of ions through the enamel into the dentin.[43] Elsewhere in this monograph Brudevold *et al.* show an increase of fluoride content in the enamel surface after fluoridation of drinking water, demonstrating that composition changes occur on the outer surface of the tooth, which may be masked by gross analysis of the enamel and dentin. Indeed, in his presentation Trautz shows that interaction of fluoride with the enamel surface produces calcium fluoride. Such changes in surface composition of the enamel may conceivably have bearing on caries susceptibility or resistance by altering the solubility of the exposed portions of the tooth.

From the foregoing, it appears that the laws regulating the relationship between inorganic precipitates and supernatant solutions[44,45] apply to tooth composition through the saliva as well as through the blood serum.

There is some evidence that the composition of the saliva may be affected by systemic factors, more specifically by factors related to blood serum composition, as shown by Shannon for 17-hydroxycorticosteroids in parotid fluid.[46,47] Shannon (personal communication) has also observed that in hyperparathyroidism (3 cases) phosphate levels are low both in blood serum and in parotid fluid. Moreover, there are indications that calcium and phosphate in the saliva, particularly phosphate, are influenced by the diet. For example, Clark[48] found that about twice as much phosphate was present in the saliva of sheep given a normal diet as in sheep receiving a phosphate-deficient diet. Buttner and Muhler[49] found an increase in salivary phosphate following oral administration of $CaHPO_4$.

The diet can also influence composition of oral fluid by direct dissolution in the saliva. Soluble Na_2HPO_4 dissolving directly in the saliva would raise its phosphate content, changing the $CO_3:PO_4$ ratio as well as the activity product of $[A_{Ca^{++}}][A_{HPO_4^{-}}]$. The change of the $CO_3:PO_4$ ratio can lead to corresponding changes in the composition of the exposed surfaces of the teeth. An increased activity product of $[A_{Ca^{++}}][A_{HPO_4^{-}}]$ would increase the saturation of the saliva with respect to the mineral salts, favoring mineral deposition while reducing solubility of the mineral deposited. Both of the above effects on salivary composition would tend to reduce solution of tooth mineral. Another facet of the cariostatic action of Na_2HPO_4 dissolved in oral fluids may be its buffering capacity, so that it could directly neutralize acids secreted by microorganisms.

Oral and Systemic Effect of High-Phosphate Diet

FIGURE 8 illustrates the cariostatic effect of dietary phosphate, via blood and saliva composition and tooth composition. The effects of diet on blood and tooth composition are fairly well established. Three questions on which experimental data are sparse are. (1) what composition change does the diet produce in the saliva? (2) to what degree is the dietary effect on saliva a systemic phenomenon? and (3) to what degree does the diet directly affect salivary composition?

Changes in the composition of saliva influence both tooth composition and

tooth solubility. Tooth solubility can be decreased by the saliva in three ways: (1) by raising the $[A_{Ca^{++}}][A_{HPO_4^{=}}]$ of the oral fluid; (2) by direct neutralization of bacterial acids and conceivably by affecting the nature of bacterial flora present in the mouth; and (3) by changing the composition of the tooth surface.

We may safely say that between the two effects of soluble dietary phosphate, namely changes in the composition of the saliva and changes in the composition of the teeth, the result is strikingly cariostatic. Which of these two effects plays a primary role in the reduction of caries by dietary phosphate remains a subject for further investigation.

Evaluation of the Proposed Caries Hypothesis

The experiments derived from our caries hypothesis based on changes in tooth composition led to the finding, confirmed in several laboratories, that soluble phosphate added to the diet is cariostatic. Careful evaluation of the findings, however, indicates that, in addition to affecting tooth composition, a direct effect of phosphate within the oral cavity, such as neutralization of acids and decreased solubility of tooth mineral, could also bring about the cariostatic result As is often the case in the history of investigative work, an hypothesis

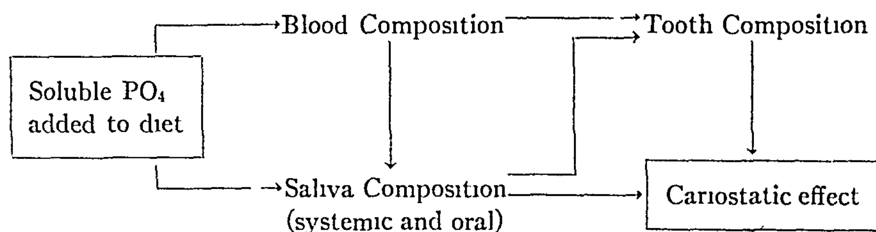


FIGURE 8 Cariostasis in relation to the composition of diet, teeth and body fluids.

leads to fruitful investigation which, however, may result in revision of the original hypothesis. We hope that future investigations will define more closely the mechanism through which the cariostatic effect of phosphate supplements to the diet is accomplished.

Summary

Studies of the composition of diet with relation to blood serum composition and the composition of the inorganic fraction of mineralized tissues led to the formulation of a caries hypothesis: high-phosphate diets produce teeth that are less vulnerable to caries because their relatively low carbonate content renders them resistant to acid attack. Experimental work in this and other laboratories has borne out the cariostatic effect of phosphate. The present interpretation of experimental evidence, however, suggests that there are two possible ways in which this effect may occur, namely, (1) increased phosphate in blood serum and saliva may alter the composition of the tooth, and (2), direct action of soluble phosphate via the saliva may reduce solubility of tooth mineral, as well as neutralize bacterial acids in the mouth.

Acknowledgment

I am indebted to Penni A. Laurence of the Jewish Hospital for her many valuable suggestions in the preparation of this manuscript.

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Enamel

There is good evidence to show that surface enamel differs markedly in character from subsurface enamel. It is harder[14,15] and contains more mineral than the underlying enamel.[16] According to results of the isotope studies, the intact enamel surface is less reactive to fluorine than are artificially ground surfaces.[17] Moreover, surface enamel has a reduced solubility as compared to subsurface enamel[7] and, what is most significant, it is more resistant to the carious process. This is shown in FIGURE 1, which represents a microradiograph of a ground section of a typical, initial, smooth enamel lesion. The enamel surface is only slightly affected, whereas marked decalcification has taken place in the enamel below it.



FIGURE 1 Microradiograph of initial carious lesion

We shall discuss some of the chemical factors that may contribute to give the enamel surface its remarkable resistance to destructive agents in the mouth. The first question to be considered is: in what respect do the three main constituents of the enamel, namely the inorganic portion, the organic portion, and the water, differ in concentration in the surface and in the body of the enamel?

A microradiographic tracing, and retardation measurements with the polarizing microscope, of intact enamel are shown in FIGURE 2. There is a slight and gradual decrease in the concentration of mineral from the surface to the dentinoenamel junction.[18] In earlier work with a coarse-grained film, a rather marked zone of hypercalcification in the surface enamel was recorded.[16] This was caused partly by an artifact, the Mackie effect, which was produced in locations of sharp contrast in the film. With the fine-grained film now avail-

INORGANIC AND ORGANIC COMPONENTS OF TOOTH STRUCTURE*

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There have been three phases of the study of tooth composition. Early work was concerned with the chemistry of the entire tooth. With the development of procedures for separating dental tissues it became possible to determine the composition of enamel, dentin, and cementum. The next logical step, the study of the chemistry of successive layers of enamel, dentin, and cementum, is the subject of this paper. In its connection the following questions may be raised. In what respect does the composition of surface enamel, dentin, and cementum differ significantly from that of the same subsurface tissues? To what extent do chemical changes in the surface layer take place with age? What is tooth maturation? How are the various surfaces affected by fluoride and other trace elements in food and drinking water? What are the components of surface enamel that strengthen it, and what is its optimal composition? These are basic questions to which we must seek the answers if we are to comprehend the many complex phenomena taking place in the tooth structure. We have worked in this field for some years; this paper summarizes our published[1-12] and unpublished findings on the composition of successive layers of enamel, dentin, and cementum.

Isotope studies have shown that surface reactions in both bone and teeth are pronounced.[13] Because of its relatively simple metabolism, the tooth is better suited for observations of surface changes than bone, which undergoes rapid alterations due to remodeling and growth. The highly calcified, nonvital enamel provides a more clear-cut picture of surface changes than does the rest of the tooth. Crown dentin, like enamel, is exposed to fluids on one side but, unlike enamel, the surface is continuously renewed because of appositional dentin formation. On the other hand, the enamel surface, which is morphologically stable, is more static and thus exposed to more prolonged exchange reactions. In the root of the tooth conditions are still more complex than in crown dentin and in many ways they may be compared to those of bone. As in long bones, we are concerned with two surfaces, the external and the internal, or pulpal and, as in bone, there is appositional growth, but without the remodeling process of bone

In the present study, pooled layer samples were obtained from the enamel, the crown dentin, and the root of a large number of teeth, and were subjected to chemical analyses. The external layers of the root were composed of cementum; the innermost layers, of dentin. Because the root was considered a metabolic entity, no attempt was made to separate cementum and dentin in the intervening layers.

* The studies reported in this paper were supported in part by Grants D-214 and D-798 from the Institute of Dental Health, Public Health Service, Bethesda, Md.

material from the dentin also may be involved.[22] From the finding of greater concentrations of organic matter and also of mineral, as previously mentioned, in the surface enamel of all intact enamel, the obvious and interesting deduction can be made that less water is present in the outermost portion than in the bulk of the enamel.

The distribution of water and of organic and inorganic material in the entire enamel is indicated in FIGURE 4. Here the distribution curve of the organic material, calculated from nitrogen, citrate, and lactate data, has been superimposed on the distribution curve of the inorganic portion obtained from radiographic tracings; for the latter a level of between 95 and 96 per cent, in accordance with most figures given in the literature, was employed. The fraction needed to make up 100 per cent is assumed to be composed of water. The procedure is certainly inaccurate in that absolute values may be in error, but it does give an indication of water distribution throughout the enamel. It is realized that water may occur in many forms, that it may be bound in the crystal lattice and in the organic matrix in addition to filling free spaces; however, in the absence of a satisfactory method of determining water in enamel and in its different layers, it is assumed that the distribution indicated in FIGURE 4 approximates that of free water. Deakins[23] has shown that the process of calcification in the enamel involves the displacement of water by minerals, suggesting an inverse relation between the extent of calcification and the concentration of water. According to this finding it may be considered that calcification is more advanced in surface and junction enamel than in the body of the enamel because more water has been displaced by mineral in these portions. The presence of relatively large amounts of organic matter has limited

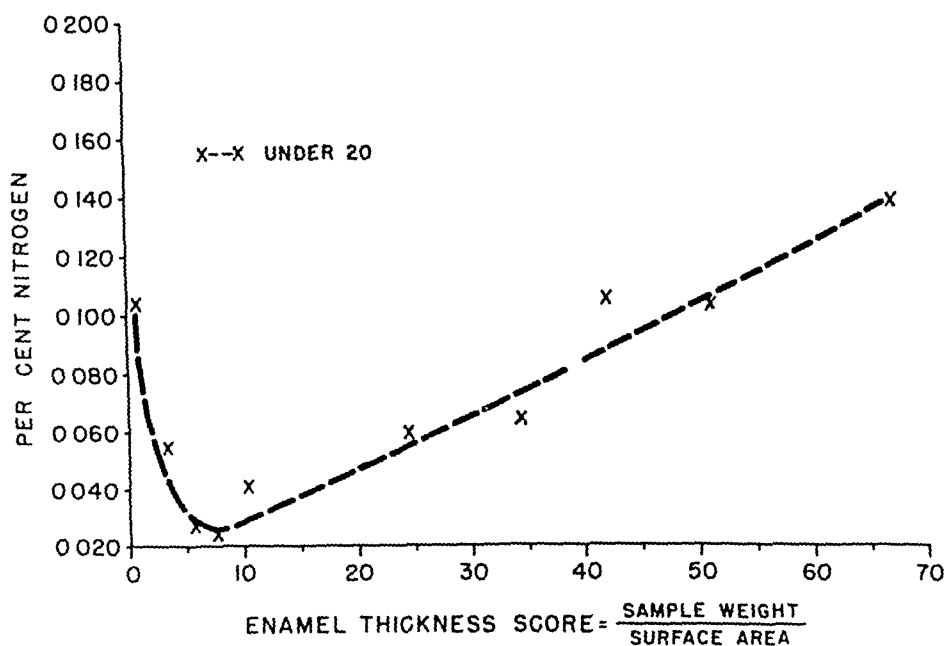


FIGURE 3 Distribution of nitrogen in enamel from the surface to the dentinoenamel junction, in erupted teeth under 20 years of age. Reproduced by permission from *The Journal of Dental Research*[11]

able, this artifact is minimal,[19] but tracings of numerous specimens of intact enamel have shown that, although the mineral concentration on the surface is not as great as previously supposed, it is still significant. From earlier microscopic studies with polarized[20] and ultraviolet light[21] it was concluded that enamel adjacent to the dentin is hypercalcified; our radiographic tracings have shown that this is not the case. The extent to which optical phenomena relate to degree of mineralization in the complex environment of the dentinoenamel junction warrants further study.

Whereas enamel mineral decreases slightly in concentration from the surface to the dentin, the distribution of organic material is more complex. As judged

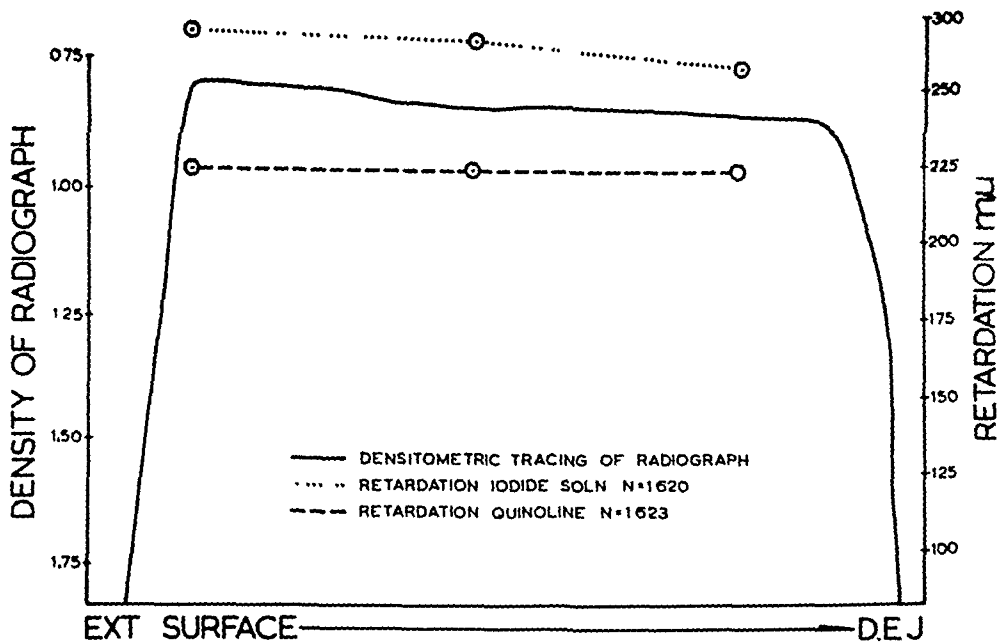


FIGURE 2 Retardations of ground planoparallel section of sound enamel and densitometric tracings of the microradiograph D E J = dentinoenamel junction Reproduced by permission from *The Journal of Dental Research* [18]

from determinations of nitrogen,[11] citrate, and lactate in successive layers, the concentrations are high in the outermost enamel, decrease sharply to a minimum in underlying layers, and then rise gradually to a maximum at the dentinoenamel junction. For the moment, only a distribution curve of nitrogen in enamel from the teeth of persons under 20 years of age will be considered (FIGURE 3). The high surface concentrations are not related to surface depositions of organic material, but are concerned with the surface enamel proper. The data were obtained by analyzing pooled layer samples of large numbers of teeth that had been thoroughly scraped prior to sampling.[11] In young teeth this high surface concentration of organic material is undoubtedly related to the presence of the primary or calcified enamel cuticle and, in older teeth inclusion of organic material from the oral cavity in the enamel undoubtedly is involved. The increase in organic material toward the dentin very likely reflects the presence of enamel tufts and spindles, and diffusion of soluble organic

material from the dentin also may be involved.[22] From the finding of greater concentrations of organic matter and also of mineral, as previously mentioned, in the surface enamel of all intact enamel, the obvious and interesting deduction can be made that less water is present in the outermost portion than in the bulk of the enamel.

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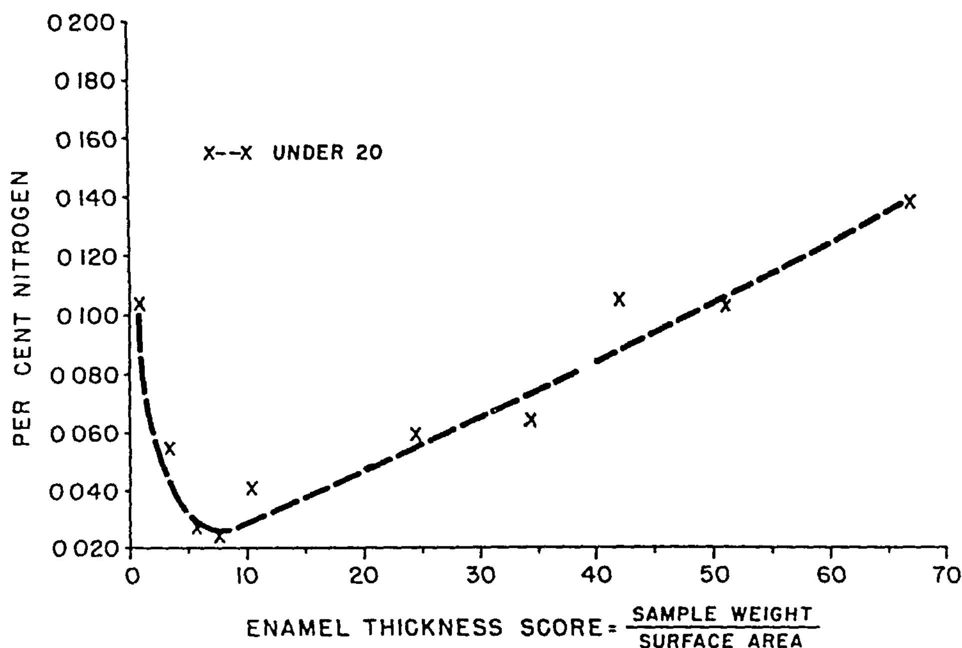


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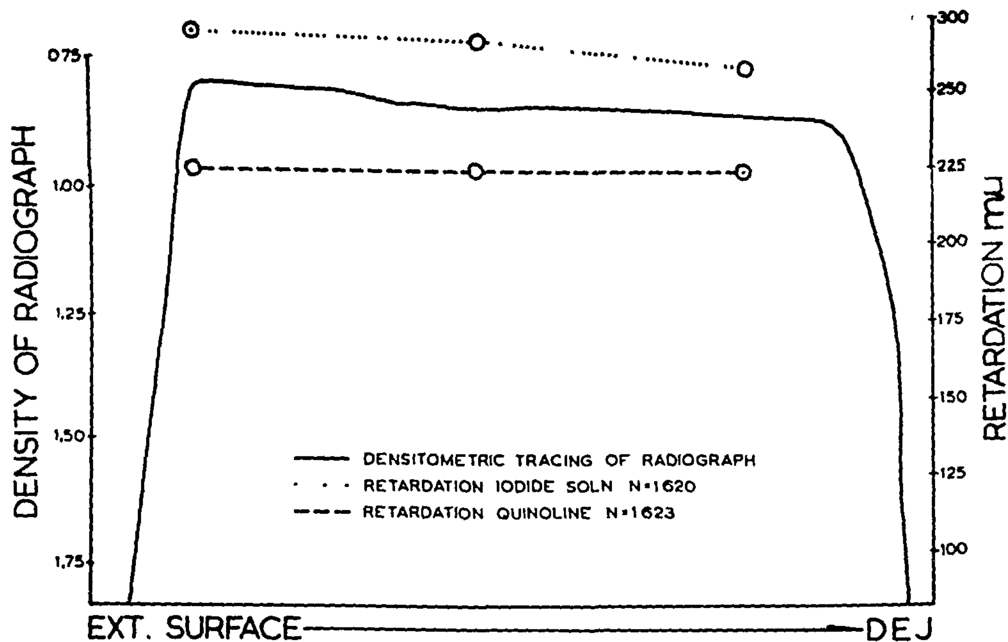


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enamel. A typical distribution pattern of F in enamel of teeth from low F areas is shown in TABLE 1. The unerupted and erupted contemporary teeth are from Rochester, N. Y., and were collected before F was added to the water supply. The ancient teeth were recovered from Indian graves in New Mexico and Kentucky.[10] The unerupted teeth, most of which were third molars, were grouped according to their stages of development (I, II, III). The first group included teeth with partly developed roots, while the third group contained teeth that were fully formed. It may be seen how F gradually builds up in the outer enamel before as well as after eruption, and how the inner enamel maintains low concentrations, not only throughout the life of the tooth but, as in the case of the buried teeth, for thousands of years thereafter.

It is suggested that the deposition of F occurs in three stages: (1) during the period of enamel calcification when F is deposited profusely, concomitantly with crystal formation; (2) after calcification is complete, but before eruption,

TABLE 1

CONCENTRATION OF FLUORINE* IN SUCCESSIVE LAYERS OF ENAMEL OF DIFFERENT AGES†

Layer	Contemporary				Ancient	
	Unerupted			Erupted	800 yrs	5000 years
	I	II	III	over 50 years		
1	331	528	847	1247	1640	2030
2	101	232	391	667	675	
3	57	150	201	404	408	542
4	33	96	172	315	232	
5			88	176	180	
6			64	147		275

* Ppm
† Brudevold *et al.*[2] and Steadman *et al.*[10]

when more F is taken up by the external surfaces of enamel from tissue fluids; and (3) after eruption and throughout the life span of the teeth, when F from the drinking water, food, and saliva is taken up by the enamel surface.

At least two factors seem to govern the rate of acquisition of F.

(1) The first is the period of F exposure, as shown in TABLE 1. The subsurface enamel becomes increasingly blocked from contact with tissue fluids as calcification proceeds, and is subjected to F exposure for only a short period of time. The surface is F-exposed both pre- and posteruptively and accordingly will acquire much greater concentrations with time.

(2) Another factor is the concentration of F in the tooth environment. With increased F ingestion there is a marked increase in the deposition of F in the surface enamel. This is shown in TABLE 2, which gives the concentrations of F in the surface and the body of the enamel of different age groups of teeth from areas with different levels of F in the water supply. In all age groups an increase in the water fluoride causes a more pronounced increase in the deposition of F in the surface than in the subsurface enamel. An increase with age of F deposition in the surface enamel is also evident in most of the tooth groups.

the accumulation of mineral, particularly in the innermost enamel, but available spaces appear to be more completely filled with mineral in the surface and junction portions of enamel than in the middle portion.

A high degree of calcification may indeed be expected in the superficial enamel because of its direct exposure to calcium and phosphate ions in tissue fluids before eruption and in saliva after eruption. Physical considerations suggest that calcification can occur only as long as a free passage of ions will permit crystal formation and growth. When the spaces between the crystals approach atomic dimensions, the movement of ions becomes increasingly restricted by charges on the crystal surfaces.[24] The rate of calcification, which initially is high, will therefore gradually slow down. Although the body of the enamel is well calcified, its contact with the calcifying medium is blocked off when the surface calcifies and only the outer enamel has the continuous contact with tissue fluids required for maximal calcification. Junction enamel also may undergo prolonged mineralization because of a relatively free ionic flow through the wide tubuli of young dentin.

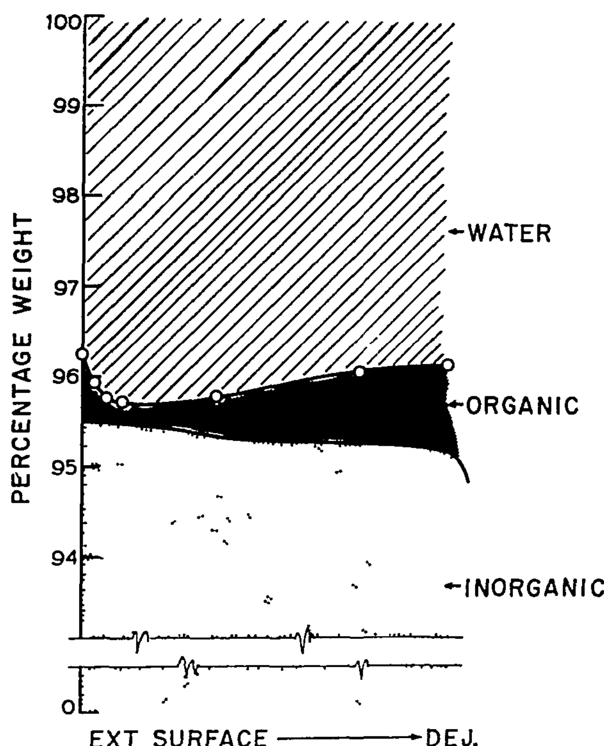


FIGURE 4. Distribution of water and organic and inorganic material in enamel from surface to the dentinoenamel junction (D E J.).

The presence of trace elements in enamel will now be considered. The findings were obtained from pooled layer samples of groups of teeth of known ages, collected from different geographic areas. Only the teeth of persons who had lived continuously in a community were included. Results[2-4,10] show that certain elements, including F, Zn, Pb, and to a lesser extent Fe, Ag, Mn, Si, and Sn occur normally in greater concentrations in surface than in subsurface

enamel. A typical distribution pattern of F in enamel of teeth from low F areas is shown in TABLE 1. The unerupted and erupted contemporary teeth are from Rochester, N. Y., and were collected before F was added to the water supply. The ancient teeth were recovered from Indian graves in New Mexico and Kentucky.[10] The unerupted teeth, most of which were third molars, were grouped according to their stages of development (I, II, III). The first group included teeth with partly developed roots, while the third group contained teeth that were fully formed. It may be seen how F gradually builds up in the outer enamel before as well as after eruption, and how the inner enamel maintains low concentrations, not only throughout the life of the tooth but, as in the case of the buried teeth, for thousands of years thereafter.

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When the difference in thickness of the enamel layers ground from the teeth is taken into account, an increase in F deposition with age was found in all groups studied. The variable results in the subsurface enamel are due to the fact that samples were obtained at different depths from the surface.

In FIGURE 5 the concentrations of F in surface and bulk enamel are plotted against concentrations of F in the drinking water. The increase in the inner

TABLE 2

FLUORINE* IN SURFACE AND INTERIOR ENAMEL OF DIFFERENT-AGED TEETH FROM AREAS WITH DIFFERENT LEVELS OF F IN THE WATER SUPPLY

F in water (ppm)	Enamel	Unerupted	Age in years			
			Under			Over 50
			20	20–29	30–49	
0.1	Surface	528	571	1205	1070	1247
	Interior	96	48	67		58
1.0	Surface		889	1087	1132	1552
	Interior		129	87	91	158
3.0	Surface		1930	2100		2290
	Interior		152	222		94
5.0	Surface	3190	3370		3350	
	Interior	793	570		858	

* Ppm

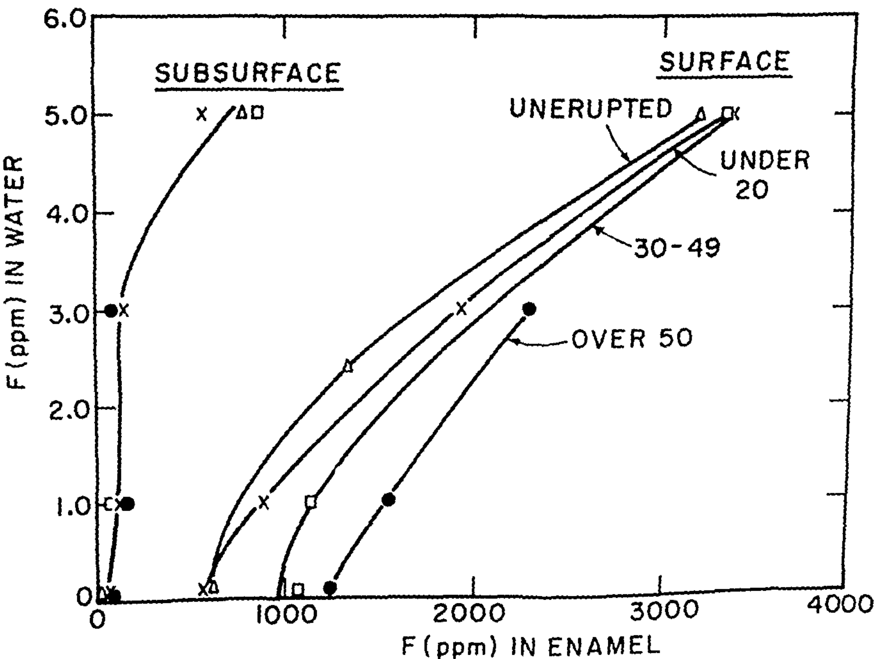


FIGURE 5. The effect of various concentrations of fluoride in the water supply on the deposition of fluoride in surface and subsurface enamel of teeth of different ages

portion of the enamel is relatively slight, while it is marked in the surface enamel and almost linearly related to the F level of the water. Since the tooth material had been collected from different communities there must have been variations in the ingestion of dietary F. However, the clear-cut relation between F deposition and the level of F in the water emphasizes the all-important effect of the water F. The increase in F deposition with age is also evident in FIGURE 5. It is most marked at low levels of F in the water and hardly noticeable at 5 ppm. At such high levels of F in the water, surface positions on the enamel crystals may be fully occupied at the time of eruption, so that

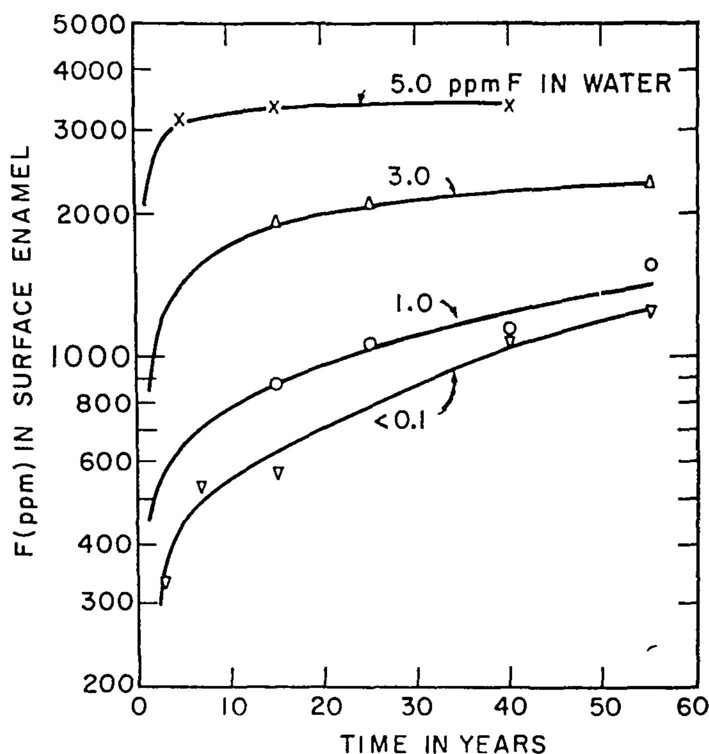


FIGURE 6 The rate of fluoride uptake by surface enamel of teeth from areas with different levels of fluoride in the water supply.

a further increase in F deposition will involve the exceedingly slow process of exchange in the interior crystal lattice.

The rate of F deposition in surface enamel is indicated in FIGURE 6. The uptake is very rapid during the first years following enamel formation and then tapers off at a level closely related to the concentration of environmental F. From the few data available on unerupted teeth from different F areas the important point can be made that the initial rate of F deposition in the external enamel is greater at high than at low levels of F in the drinking water.

These comments on F deposition essentially apply also to lead[3] and possibly to zinc. TABLE 3 shows that both unerupted and erupted teeth have high concentrations of lead in the external enamel and much lower concentrations in subsurface enamel. There is also an increase of lead with age, which is much more marked in the external than internal portion of the enamel. It has been found that concentrations of lead in surface enamel vary by a factor

of at least 2 in teeth from different parts of the United States, and that the tooth enamel of primitive people from two Pacific islands and from Greenland contains smaller concentrations of Pb than does enamel from the United States.

Next to F, Zn is the trace element that occurs in greatest concentrations in enamel. Surface enamel contains at least two or three times more Zn than does internal enamel. As shown in TABLE 4, Zn concentrations may vary considerably in teeth from different areas. Most of the Zn appears to have been deposited in the enamel before eruption. Although zinc is known to occur in many proteins, it is probably deposited primarily in the inorganic portion of the enamel; this is suggested by our observation that Zn will combine readily with synthetic hydroxyapatite. FIGURE 7 shows the rate of uptake of Zn by 50 mg. synthetic hydroxyapatite from 10 ml. of a solution having the same concentration of Zn and the same ionic strength and *p*H as blood. The rate of uptake is of the same order as that of F and other bone-seeking elements. This finding and the distribution pattern with high surface concentrations suggest that Zn, like Pb and F, reacts with the apatite in enamel crystals. The mechanism involved in the uptake of Zn by enamel and the significance of its presence have not been studied. It has been suggested that the teeth of tuberculous patients contain more Zn than do those of healthy persons.[25]

TABLE 3
LEAD* IN SUCCESSIVE LAYERS OF ENAMEL†

Layer	Unerupted	Erupted			
		Under 20 years	20–29 years	30–49 years	Over 50 years
1	210	350	360	520	550
2	130	220	260	430	460
3	67	180	105	280	420
4	60	74	85	200	310
5	55	35	65	—	156
6	47	—	54	—	152

* Ppm.
† Brudevold & Steadman.[5]

TABLE 4
ZINC* IN LAYERS OF ENAMEL OF ERUPTED AND UNERUPTED TEETH

Layer	Schenectady, N. Y.; unerupted	Augusta, Me.			Tonga Islands, age unknown
		Unerupted	Under 20 years	30–49 years	
1	1700	1500	2100	1500	1070
2	1600	930	1200	1100	590
3	630	930	1100	900	470
4	400		870	770	470
5	300		700	730	470
6	180	610	730	670	390

* Ppm.

Since the concentrations of Zn vary greatly in teeth from different communities, it is likely that geographical variations rather than the incidence of tuberculosis contributed to these findings.

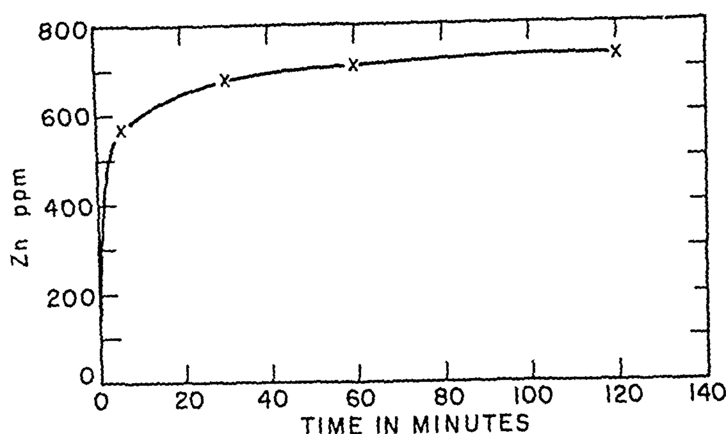


FIGURE 7. The rate of uptake of Zn^{++} by hydroxyapatite from a solution containing 4 ppm of Zn^{++} at *p*H 7.4 and ionic strength 0.16.

FIGURE 8. The distribution of CO_2 (carbonate) in enamel from the surface to the dentino-enamel junction Reproduced by permission from *The Journal of Dental Research*[8]

While the accumulation of many trace elements in surface enamel is important, the fact that certain enamel constituents have a reverse pattern of distribution and are present in lower concentrations in surface than subsurface enamel may be of equal significance. These constituents include carbonate,[8] Na, and Mg. FIGURE 8 shows how the carbonate increases from the surface

inward. This interesting pattern of distribution occurs in both unerupted and erupted teeth and must be established during the enamel formation. The mechanism involved is not known. It is possible that the carbonate: phosphate ratio in the enamel organ decreases as calcification advances, but this has not been proved.

Carbonate concentrations in the external enamel tend to decrease with age, while no changes have been observed in the body of the enamel. This finding, shown in FIGURE 9, was unexpected because carbonate in bone is known to increase with age[26] and it was thought that the high concentrations of carbonate in saliva might cause a similar increase in enamel carbonate. At least two factors must enter here. (1) carbonate is known to be preferentially dissolved

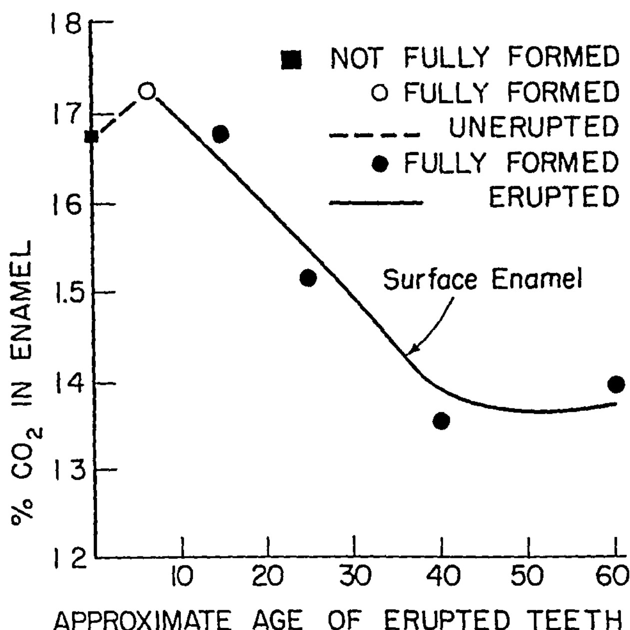


FIGURE 9 Changes with age in the concentration of CO_2 in surface enamel Reproduced by permission from *The Journal of Dental Research*[8]

from enamel by weak acids,[27] and (2) conditions in the mouth may favor an exchange of carbonate with phosphate and other anions. According to Sobel, a decrease in carbonate concentration of enamel is related to an increased resistance to dental caries,[28] but this possibility and the mechanism of the peculiar distribution of carbonate in enamel warrant further study.

A number of elements, including Sr, Cu, Al, and K have no definite distribution pattern in enamel and appear to occur in approximately the same concentrations in surface and subsurface enamel.[1,9] The distribution of Sr in the enamel of unerupted and erupted teeth from different geographical areas is shown in TABLE 5. The principal acquisition occurs before eruption, probably during tooth formation, there appears to be no change in concentration with age. However, teeth from different geographical areas vary considerably in Sr levels. The findings parallel those in bone, and the range of concentrations is also similar in the two tissues.

The concentration of Cu in enamel is less than that of Sr and, like Sr, it is of the same magnitude as that found in bone. TABLE 6 shows the concentration of Cu in successive layers of enamel in unerupted teeth and erupted teeth of different ages. These and other data suggest that the copper is deposited during calcification and that no significant changes in concentration take place after eruption. The findings given in TABLE 6 were obtained from pooled layer samples ground from twenty or more teeth. The data on Cu in the enamel of individual teeth showed no correlation with tooth discoloration, a plausible possibility in view of the observation that trace amounts of Cu in protein may catalyze formation of enzymatic pigment.[29]

TABLE 5
STRONTIUM* IN SUCCESSIVE ENAMEL LAYERS IN TEETH FROM DIFFERENT GEOGRAPHICAL AREAS†

Layer	Augusta, Me			Big Lake, Tex, 20–29 years	Clovis, N Mex, 30–49 years	Tonga Islands
	Unerupted	Under 20 years	30–49 years			
1	100	120	105	270	25	320
2		120	110	260		400
3		125	125	230	42	470
4	100	140	112	260		450
5		110	150	260		600
6	110	80	125	350	100	320

* Ppm
† Steadman *et al* [9]

TABLE 6
COPPER* IN SUCCESSIVE LAYERS OF ENAMEL†

Layer	Unerupted	Erupted			
		Under 20 years	20–29 years	30–49 years	Over 50 years
1	7	20	17	20	20
2	7	15	26	20	22
3	7	30	25	20	13
4	5	25	26	18	12

* Ppm.
* Brudevold & Steadman [1]

Reference has been made to the high concentrations of organic material in surface enamel. According to our findings, there is no measurable increase with age in the N concentrations of surface enamel until the last decades of life. In a study of N in the surface enamel of teeth of different age groups, only those over fifty years showed greater concentrations of N than younger teeth. A more clear-cut increase in N with age has been found in the outer portion of enamel by relating N to volume,[30] rather than to weight, as was done by us Theoretically, there is little reason for believing that the organic material in surface enamel should increase with age, were it not for the wear and

tear to which the dentition is subjected. The increasing number of cracks in old teeth and the voids created by partial decalcification are apt to be filled with salivary matter that cause an increase in organic concentrations. However, in enamel, old or young, without such defects, this increase is unlikely because spaces and pathways probably are too narrow to permit appreciable passage of large organic molecules.

FIGURE 10 gives the concentrations of N (dry weight) in successive layers of enamel from the surface to the dentin in groups of teeth of different ages. The oldest group, over fifty years, differs from the younger groups in three respects: (1) as already mentioned, greater concentrations of N are present in the surface enamel of the oldest teeth; (2) greater concentrations also occur at the dentinoenamel junction, and (3) the minimal level in the body of the enamel extends farther in depth than in the younger teeth. The decrease in N in the middle portion of old as compared to that in young enamel is believed to be associated with an increase in mineral, possibly brought about by the many cracks in these teeth. It has been shown that the cracks will fill with organic material and eventually calcify. Enamel adjacent to the cracks may also acquire calcium salts from saliva and undergo further calcification A progressive calcification of this type has been observed in hydroxyapatite exposed to calcifying solutions[31] and may generally be expected in incompletely calcified material in contact with solutions containing high concentrations of Ca and phosphate at approximately neutral pH Saliva diffusing through the cracks may thus gradually be deprived of mineral, and as a result more organic than

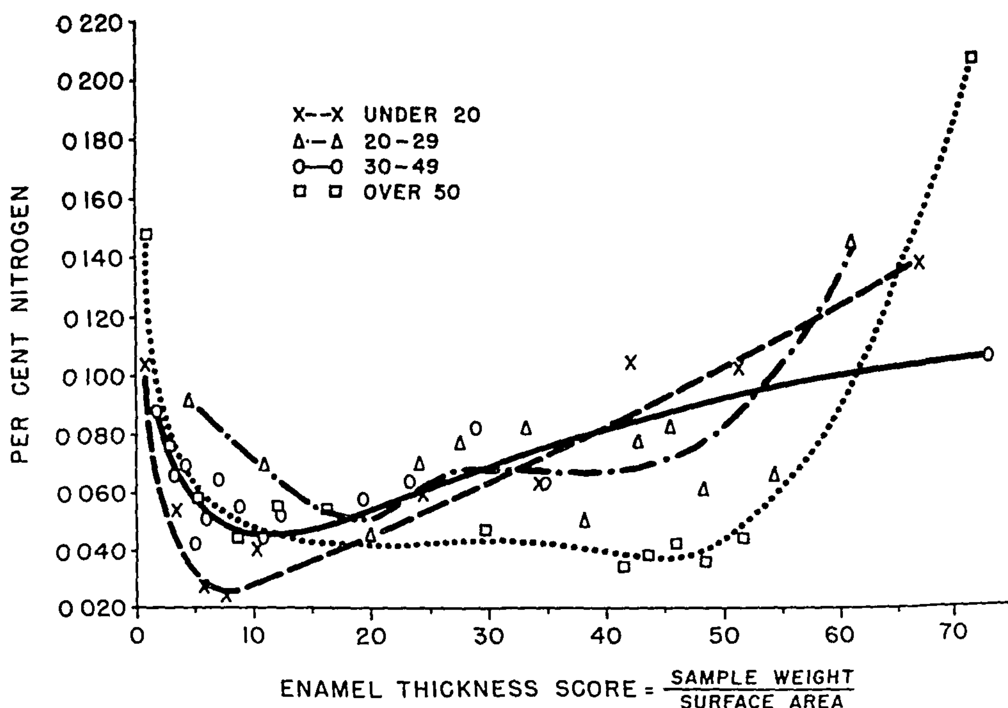


FIGURE 10 Distribution of nitrogen in enamel of teeth of different ages Reproduced by permission from *The Journal of Dental Research*[11]

inorganic material may deposit in the innermost portion of the enamel; this is suggested by the increase in N in the junction enamel of old teeth as compared to that in young teeth.

As previously mentioned, citrate and lactate occur in greater concentrations in surface and junction enamel than in the body of the enamel. This is shown in FIGURE 11 in which molar concentrations in successive layers of enamel from a group of erupted teeth are plotted against the distance from the enamel surface. The citrate figures are lower than would be expected from the data on total enamel given in the literature.[32,33] It is not known to what extent this is due to biological variation or to difference in procedures. Because of the great difference in concentration in different portions of the enamel, methods of sampling obviously will influence results. A possible variable in connection

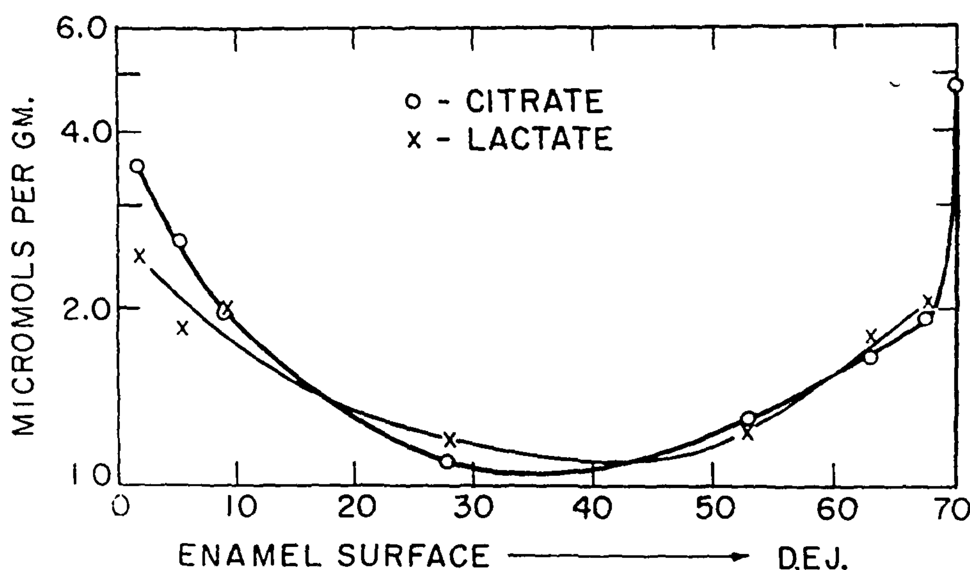


FIGURE 11 The distribution of citrate and lactate (in micromoles per gram) in surface and subsurface enamel of teeth from persons under 20 years of age D.E.J. = dentinoenamel junction

with both citrate and lactate, that is, difference in deposition with age, has not yet been studied.

The presence of citrate in enamel is not surprising, since it is readily taken up by hydroxyapatite.[34] However, we have found that only insignificant amounts of lactate are acquired by apatite at physiological *p*H. It is possible that both citrate and lactate are located primarily in the water in the enamel, since a comparison of the distribution curves with the curve of water (FIGURE 4) shows striking similarities.

Many organic compounds have been found in enamel, including carbohydrate, proteins, fat, and less complex substances. Stack recently has reported at least ten times more carbohydrate in the outermost layer than in the body of the enamel,[35] which suggests that the major portion of the protein in the surface enamel is composed of glycoprotein Aside from Stack's study, work on organic material in enamel has been concerned chiefly with the entire enamel and will not be reviewed here.

Considerable significance has been attributed to the organic material in surface enamel. It has been claimed to provide protection of the enamel against decalcification; the inclusion of protein and the change in the nature of the protein have been suggested as important factors in the maturation of the enamel. The findings summarized in TABLE 7 suggest that the lesser solubility of surface as compared with that of subsurface enamel is due to differences

TABLE 7

SOLUBILITY RATE OF ENAMEL BEFORE AND AFTER REMOVAL OF ORGANIC CONSTITUENTS

Fifty-mg Samples of a First and Sixth Layer of Enamel Exposed to 5 ml of 0 5 *M* Acetate at *p*H 4.0 for 1 hour*

	Control		Ethylenediamine-extracted	
	1st layer	6th layer	1st layer	6th layer
Loss of wt. (%)	37.2	45 7	33 9	40 7
Loss of Ca (mg)	6.77	7 93	6.42	7 50
Loss of P (mg)	3 15	3.75	2 83	3.54

* Isaac *et al.*[7]

TABLE 8

RANGE OF CONCENTRATION IN MICROMOLES PER GRAM OF DIFFERENT CONSTITUENTS OF SURFACE ENAMEL AND THE BODY OF INTACT ENAMEL OF TEETH FROM DIFFERENT GEOGRAPHICAL REGIONS

	Outer enamel	Body enamel
Carbonate	440 0–350 0	654 0–525 0
Na	360 0–230 0	380 0–310.0
F	176 0–17 0	44 0–3 8
Mg	60 0–30 0	74 0–60 0
Zn	27 5–6 6	14 2–2 9
Citrate	5 0–3 5	1 1–
Al	4 8–1 4	4 5–1 1
Sr	3 7–0 3	4 6–0 7
Lactate	2 9–	1 2–
Pb	2 6–0 4	1.1–0 1
Cu	1 8–0 1	0 6–trace
Si	1 5–0 2	1 8–0 1
Ag	0 9–trace	0 5–0 0
Fe	0 6–0 4	0 4–0 2
Sn	0 4–trace	0 3–0 0
Mn	0 4–0 1	0 2–0 1

inherent in the inorganic portions of outer and inner enamel, and that the organic matter is of little contributory significance. The organic matter was removed from aliquot layer samples by ethylenediamine extraction. Both with and without the presence of organic material, surface enamel was less soluble than subsurface enamel The consistent decrease in solubility of the enamel treated with ethylenediamine is believed to be due to crystal growth and a resulting decrease in surface area

These findings give some evidence of the importance of the composition of the mineral portions of the enamel TABLE 8 gives the range of concentrations

of some of the mineral constituents of surface enamel of teeth from different geographical areas. For purposes of comparison, concentrations are given in micromoles per gram rather than in parts per million. F and Zn may be considered as among the major components of surface enamel because they have been found to occur in considerably higher molar concentrations than other trace elements on which we have data. It will be noted that F may vary tenfold in concentration in different specimens of surface enamel—a considerably greater range of variation than has been found for other major constituents.

Crown Dentin and Root

Compared to the information available on enamel, little is known about the chemistry of the surface and body portions of the crown dentin and root.

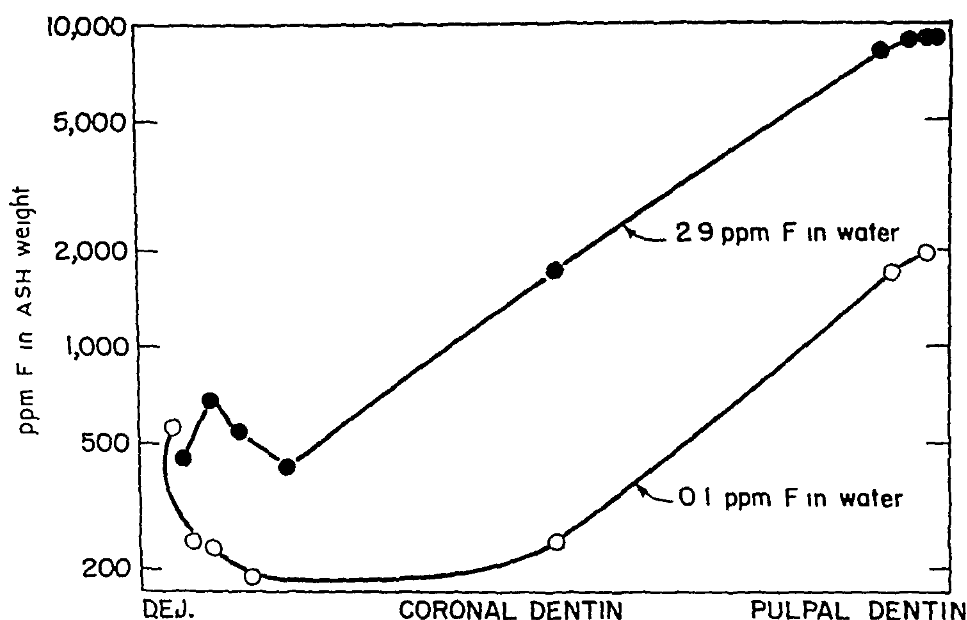


FIGURE 12 The distribution of F in coronal dentin from the dentinoenamel junction (D E J) to the pulp in teeth over 50 years of age from communities with 2.9 and 0 1 ppm of F in the water

The only references found in the literature other than our own are those of Jenkins and Speirs[36] and Speirs,[37] who found greater concentrations of F in the dentin adjacent to the pulp than in the body of the crown dentin. Our own work[5,9] is limited to observations on F, Pb, Zn, Sr, and findings dealing with lactate and citrate. The distribution of these constituents in crown dentin and roots will now be considered.

The concentrations of F from the dentinoenamel junction to the pulp are plotted in FIGURE 12, in which are shown the data from two groups of teeth over 50 years of age from communities with, respectively, 0.1 and 2.9 ppm of F in the water supply. There is a marked difference in the F level in the dentin of the two groups, but the distribution curves are strikingly similar in shape except in the portions in proximity to the enamel. A study of several groups of teeth has shown that the F level may be irregular and may increase or decrease in the first layers from the junction inward, but then a consistent in-

crease occurs which, as shown in FIGURE 12, persists all the way to the pulp. It should be mentioned also that junction dentin always contains 3 or 4 times more F than does junction enamel.

There is no change with age in F in the crown dentin, except in the portion approaching the pulp, which shows a marked increase. For example, in teeth under 20 and over 50 years old from one community the junction and middle portions of the crown dentin contained concentrations of F ranging from 700 to 1200 ppm, whereas the pulpal portion of dentin of teeth under 20 years of age contained 3400 ppm of F and that of teeth over 50 years old, 6600 ppm.

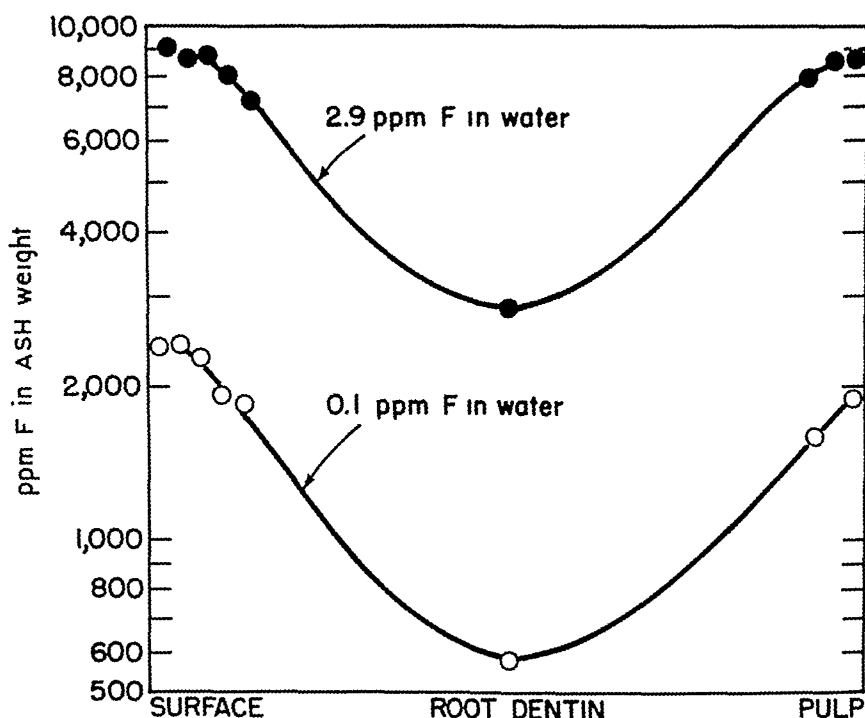


FIGURE 13 The distribution of F in roots from the surface to the pulp in teeth over 50 years of age from communities with 2 9 and 0 1 ppm of F in the water supply

The distribution of F in the roots of the previously described groups of teeth is given in FIGURE 13. Again, there is a marked increase in F deposition with an increase of F in the water supply, and again the distribution curves are strikingly similar in shape. Concentrations of F are high in the cementum, decrease to a minimum about half way to the pulp, and then increase to a level similar to that of cementum in the dentinal portions approximating the pulp. An increase in F deposition with age was found to be marked in the cement and pulpal portions and less pronounced in the deepest layers, whereas there was no increase in the middle portion except in the first decades of life.

The distribution of Pb and Zn in crown dentin and roots is given in FIGURES 14 and 15; the crown data are from teeth over 50 years old, the root data from unerupted teeth. Both elements accumulate in the surface layers, but the surface concentrations of Zn are much more pronounced than are those of Pb. The shape of the distribution curves is essentially the same as for F in crown

dentin as well as in roots. The data available are insufficient to determine the effect of age on the deposition of Pb and Zn in dentin and cementum. However, it is our impression that an equilibrium with tissue fluids is approached much sooner than in the case of F, and that the marked increase found for F does not take place.

The distribution of Sr in roots of teeth from different geographical areas is given in TABLE 9. There is no significant difference in concentration in surface

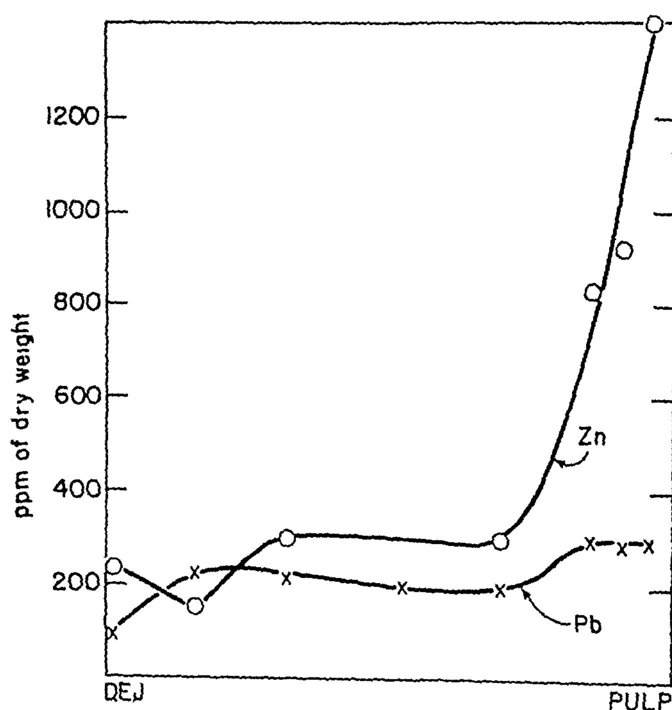


FIGURE 14. The distribution of Pb and Zn in crown dentin from the dentinoenamel junction (D E.J) to the pulp of teeth over 50 years of age.

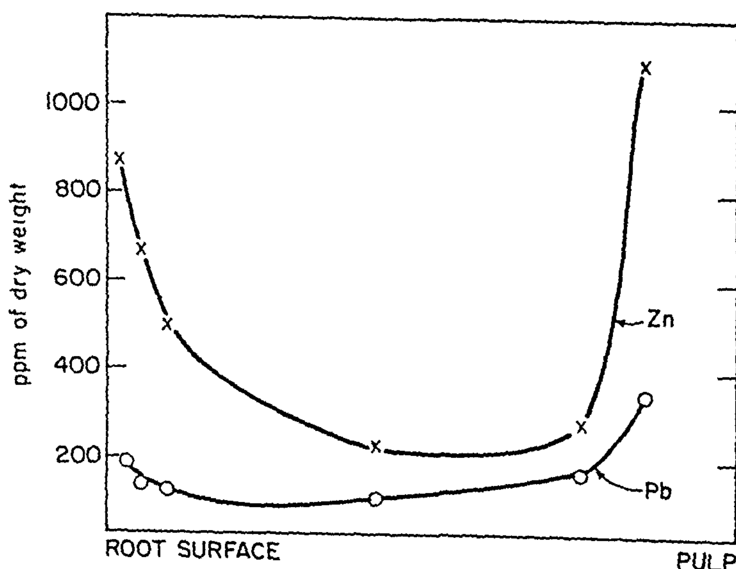


FIGURE 15. The distribution of Pb and Zn in roots from the surface to the pulp of unerupted teeth from Schenectady, N Y.

crease occurs which, as shown in FIGURE 12, persists all the way to the pulp. It should be mentioned also that junction dentin always contains 3 or 4 times more F than does junction enamel.

There is no change with age in F in the crown dentin, except in the portion approaching the pulp, which shows a marked increase For example, in teeth under 20 and over 50 years old from one community the junction and middle portions of the crown dentin contained concentrations of F ranging from 700 to 1200 ppm, whereas the pulpal portion of dentin of teeth under 20 years of age contained 3400 ppm of F and that of teeth over 50 years old, 6600 ppm.

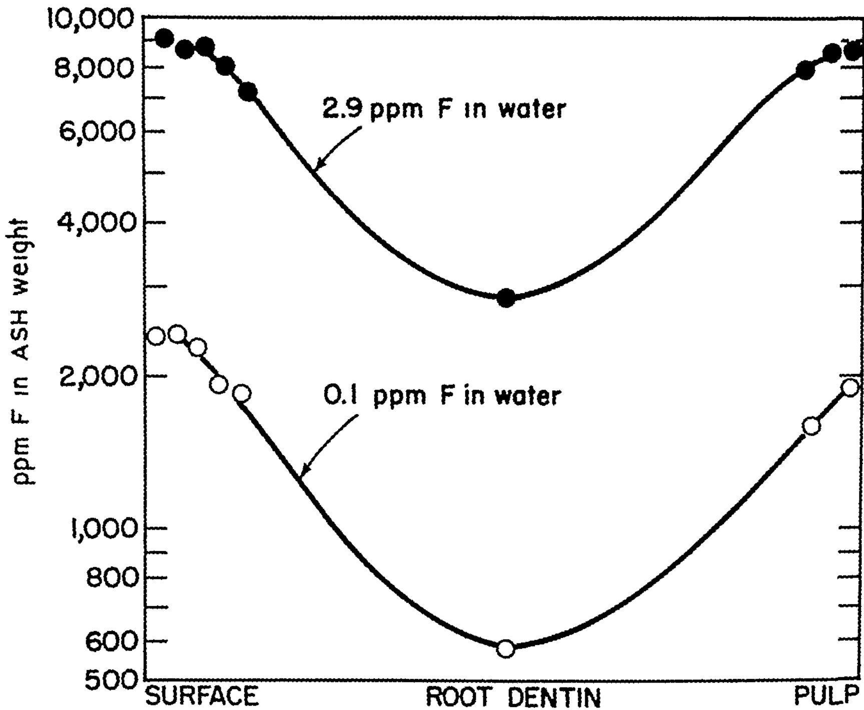


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samples have repeatedly shown concentrations of F about two times greater in dentin than in enamel. F determinations of layer samples, on the other hand, reveal that surface enamel may have concentrations up to five times greater than the body portion of crown dentin and, in one group of teeth, the layer of dentin adjacent to the pulp contained more than eighty times the concentrations found in the inner portion of enamel. With such marked differences it is obvious that the composition of surface layers is of vital importance in the study of trace elements in dental structures.

The gradation of F was more abrupt in enamel than in the other tooth portions. Differences in the rate of development of enamel, dentin, and cementum may account to a large extent for this difference in patterns of distribution. Enamel is formed over a relatively short period, and only the surface is subjected to prolonged F exposure. The continuous formation of dentin and cementum, on the other hand, will allow contact with tissue fluids of different portions of these structures for extended periods. Cement formation is known to be slow, whereas dentin is formed rapidly in the early stages of tooth development and at a slow rate after the root has been completed. The distribution of F in these tissues suggests an inverse relation between the deposition of F and the rate of formation. However, the diffusion of F from the surface inward must also contribute to the deposition of F, but to a lesser extent in enamel than in dentin: only the outer layers of enamel show an increase in F concentration in a lifetime. On the other hand, appreciable penetration of F appears to occur in newly formed and young dentin, since the deeper layers of dentin show maximal F increase in the first decades of life. The principal mechanism of F deposition is the exchange of F for hydroxyl groups.[38] The fixation of Pb, Zn, and many other trace elements in dental structures is probably dependent on the rate of tissue formation and permeability, as is the case with F, but our knowledge about the mechanism of deposition of these elements is incomplete.

The chemical characteristics that distinguish the surfaces of enamel, dentin, and cementum from their underlying layers are very likely of great clinical significance. For example, there can be little doubt that the high concentrations of F in the surface enamel bring about its caries-reducing action. From our data it appears that the addition of 1 ppm of F to the drinking water causes an increment of about 300 ppm or 16 μM/gm. in surface enamel over that normally occurring in teeth from low-F areas That enamel is affected and acquires caries resistance by such a small F increase must be due to its crystalline structure. Since the surface area of enamel crystals is small and only partly accessible, minute quantities of anions and cations are required to fill available surface positions. However, these strategically placed surface ions, although few in number, may affect markedly the properties of the entire crystal. For example, the mechanism of caries reduction by F probably involves a reduction in solubility, as the increase in solubility of successive enamel layers parallels the decrease in F concentrations from the surface inward.[7] The decrease in enamel hardness from the surface inward may also be an effect of F, since exposure of enamel surfaces to F increases their hardness.[39] Carbonate may affect enamel in a way opposite to that of F, and may increase solubility and caries susceptibility The significance of other ions that occur in different

and interior layers. Analysis of crown dentin gave similar results, the levels of Sr being comparable to those found in corresponding samples of enamel. Our findings suggest that the main acquisition of Sr occurs before tooth eruption, probably during tooth formation. Additional deposition of Sr takes place in the secondary dentin and cementum formed posteruptively, this must be true because in older teeth the outer layers and the dentin adjacent to the pulp contained concentrations of Sr similar to that of other portions. It follows that, in regard to the deposition of Sr^{90}, the possibility of appreciable preeruptive and of some posteruptive acquisition must be considered.

Citrate appears to be distributed evenly in crown dentin and roots except for the portion adjacent to the pulp, which consistently has been found to contain approximately two times greater concentrations Cementum and the body of crown and root dentin have given values ranging from 7000 to 8000 ppm, while two successive pulpal layers contained about 14,000 ppm These findings, from both unerupted and erupted teeth, suggest a possible relation between high concentrations of citrate and the process of dentin formation.

TABLE 9
STRONTIUM* IN SUCCESSIVE LAYERS OF ROOTS OF TEETH FROM DIFFERENT GEOGRAPHICAL AREAS†

Layer	Big Lake, Tex, 20–29 years	Doland, S.D.; under 50 years	Augusta, Me		Tonga Islands
			Under 20 years	30–49 years	
1	200	100	120	165	620
2	460	120	128	200	520
3	330	120			
4		110			
5		230			
Bulk	450	160			

* Ppm.
† Steadman *et al* [9]

Lactate was found to have a distribution pattern different from that of citrate. It occurred in the greatest concentrations in the surface of the cementum, where it ranged from 600 to 900 ppm, as compared with only about 200 ppm in the middle portion and about 300 ppm adjacent to the pulp Generally, these findings show that both inorganic and organic constituents are unevenly distributed in the surface and body of dentin, as in enamel.

Comments

Except for the F data, the present analyses were given in parts per million of dry weight. With conversion to ash concentrations, the surface layers of cementum and dentin generally contained higher levels of trace elements than did the surface layers of enamel, as might have been expected in view of the smaller crystal size and greater surface area of cementum and dentin. However, all portions of dentin do not always contain levels of trace elements higher than those in enamel. Some F data illustrate this point. Analyses of bulk

Ag, Fe, and Sn are normally present in concentrations below 10 μM/gm. and are therefore less likely to affect the physical properties of dental structures.

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concentrations in surface and subsurface enamel, including those of Na, Mg, Zn, and citrate, has not been established, but the intriguing possibility exists that future preventive treatment will aim at both enriching the enamel surface and depriving it of some of its constituents.

Nothing is known about the biological significance of differences in composition of the surfaces of cementum and dentin. It is not unlikely that the composition of the surface affects the activity of adjacent cells and hence such phenomena as appositional growth of cementum and dentin root resorption and even the functional relation between the cementum and the periodontal membrane.

The importance of enamel maturation is often discussed in the literature, but no attempt has been made to determine what the process is. The present findings show that changes occurring in the enamel after it is calcified are confined to the outer portion and that these changes to a great extent are concerned with surface accumulation of trace elements It is suggested that maturation involves these surface changes, and that the acquisition of F, which has been shown to occur at a particularly rapid rate in the pre-eruptive period, is an important phase of it. Delayed tooth eruption may indeed be desirable, since it permits maximal inclusion of F in the enamel surface before it is exposed to the hazards of the oral cavity. Maturation may also involve deposition of mineral, as a greater uptake of P^{32} has been noted in unerupted than in erupted teeth.[13] The loss of water associated with mineral deposition, and F impregnation, must be important processes in strengthening the enamel.

Summary

Microradiographic tracings of intact enamel demonstrated a slight decrease in total mineral concentration from the surface to the dentinoenamel junction. Determinations of N, citrate, and lactate in successive layer samples, pooled from 20 to 40 teeth, showed decrease from the surface inward in the outer one quarter to one half of the enamel and then increase to a maximum at the dentinoenamel junction. The distribution of organic and inorganic constituents suggests that water is most sparse in surface and junction enamel and that it occurs in greater amounts in the body of the enamel. Analysis of layer samples of enamel, dentin, and cementum showed greatest concentrations of F, Pb, Zn, Sn, Fe and, to a lesser extent, Al and Si in surface enamel, outer cementum, and dentin adjacent to the pulp. In ancient teeth exposed to soil for several thousand years the surface accumulation was greater than in contemporary teeth, while concentrations in interior material remained low. Tooth material from different geographical areas varied in composition The concentration of F in surface enamel of similarly aged groups of teeth increased nearly linearly with increasing levels of F in the drinking water, whereas the increase in the bulk of the enamel was appreciable only when the water F exceeded 3 ppm. The superficial portions of all dental structures acquired increasing amounts of F with age, while the subsurface material remained virtually unchanged. In enamel, certain constituents, including carbonate, Na, and Mg increased in concentration from the surface inward while others, such as Cu and Sr, were evenly distributed. Among the trace elements only F and Zn occur in relatively large concentrations. Other elements, including Al, Sr, Pb, Mn, Cu, Si,

CHANGING CONCEPTS IN DENTAL HISTOLOGY

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In the decade and a half during which the electron microscope has been applied to the examination of calcified tissues considerable information about the microstructure of teeth has accumulated. The type of studies carried on and the technical means by which the data have been derived reflect directly the progress that has taken place in the field of electron microscopy itself. For the first few years the studies were fairly well limited to those that could be made through use of the replica techniques, and the primary concern was with the structure of fully calcified enamel and dentin. When methods for the direct sectioning of soft tissues were introduced about ten years ago, the scope of the investigations was greatly extended, for it became possible to examine embryonic material as well as mature specimens that had been demineralized. Continuous refinement has been made in sectioning technique, until today we are approaching the point where even fully calcified specimens can be cut routinely.

It is inevitable that the observations made in such investigations with the electron microscope will bring about revisions in the histological concepts established earlier through light microscopy. The present discussion will center upon three general areas in which the new evidence is most impressive. The first concerns the interrelationships between odontogenic cells, with special attention directed to terminal bars and desmosomes. The second relates to the origins of the organic matrices of enamel and dentin, with the principal emphasis placed on fibrogenesis. The third concerns the structure of mature enamel and dentin; here the points of major interest are crystal structure in the former and the so-called peritubular region in the latter.

Interrelationships Between Odontogenic Cells

Early in the application of the electron microscope to cytological studies it was realized that the preparatory methods employed in optical microscopy could not be adapted easily for use with this instrument. The consequent developments in fixation, embedding, and microtomy are well known, and it is primarily through the resultant improved tissue preservation that new information on the interrelationships between the odontogenic cells has been forthcoming.

When tooth germs are fixed successfully with osmium tetroxide, very little space exists between either the odontoblasts or the ameloblasts, except in regions where intercellular fibrils are present.[1-3] The cell membranes of the odontoblasts occasionally show minute indentations and projections (FIGURE 1), but there has been no evidence of interconnections between cells or special structural configurations at contacting regions of adjacent cell membranes. In our present work on amelogenesis extreme folding of the lateral cell membranes of the ameloblasts has been observed but, whether straight or folded, adjacent membranes have closely paralleled each other (FIGURES 2 and 3).

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Desmosomes also have been seen at various points along the laterally contacting basal ends of the ameloblasts, as well as along the line of contact between the ameloblasts and the cells of the stratum intermedium (FIGURE 5). In addition, new information has been obtained regarding the terminal bar systems, which have been described at the optical level for some time.

In the generally accepted concept of amelogenesis, matrix formation begins with the elaboration of the so-called dentinoenamel membrane, following which a system of terminal bars and Tomes's processes is developed at the distal ends of the ameloblasts.[4,5] It is thought that these terminal bars are the source of the interrod material and that the Tomes's processes give rise to the rod substance proper. Matrix production is supposed to occur rhythmically, new terminal bars and Tomes's processes forming every few microns as the ameloblasts migrate from the dentinoenamel junction. A second row of terminal bars has also been seen optically at the opposite, or basal, ends of the ameloblasts, but even at the optical level the two terminal-bar systems have not appeared structurally identical.

An adequate explanation has never been offered as to why the terminal bars in the distal region of the ameloblasts, unlike those in other epithelial tissues, are transient, being present only during matrix production. Likewise, the problems of how and why, in this unique instance, the function of the terminal bars changes from supposed cellular cementation to matrix formation have remained unsettled. In recent studies at the electron microscopic level, we have observed that only the structures in the basal ends of the ameloblasts resemble the terminal bars that have been seen in other tissues of epithelial origin (FIGURES 4 and 6). On the other hand, the structures optically designated as terminal bars at the distal ends of the ameloblasts have appeared to be actually extracellular material identical in character to young enamel matrix (FIGURE 7). The identification of these deposits simply as enamel matrix affords a less complicated explanation of amelogenesis and, at the same time, effectively dismisses the questions and problems that have arisen as a result of their interpretation as terminal bars.

Origins of the Organic Matrices of Enamel and Dentin

The origins of the organic matrices of enamel and dentin are still not clearly understood, and it is likely that, as in the past, concepts of the mechanisms involved will continue to be derived by deductive reasoning from visual evidence. To this end, some pertinent data have been obtained through electron microscopy.

The collagenous portion of the developing dentinal matrix has been investigated quite extensively and systematically.[2,6,7] Two groups of fibrils, both bearing the 640 Å cross bandings of typical collagen from their first appearance, have been seen.[2] The first fibrils formed make up the so-called Korff's fibers, which constitute the major component of the mantle predentin. These fibrils are found between the odontoblasts, terminating in a fanlike arrangement in the region between the ends of the cells and the future dentinoenamel junction (FIGURE 8). They increase in number during elaboration of the mantle predentin. After this layer, only a few microns thick, has been formed, the pro-

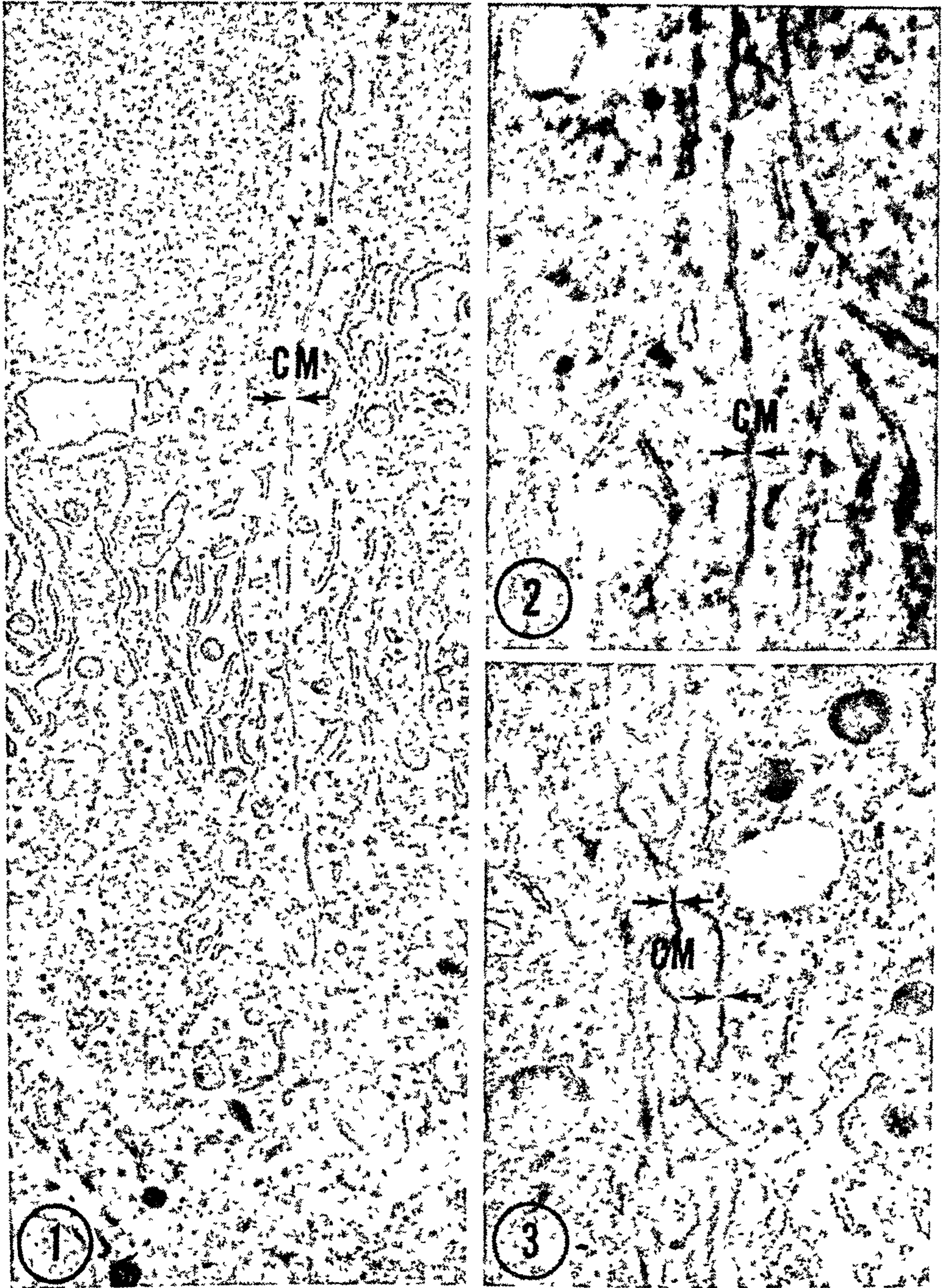


FIGURE 1. Cell membranes (*CM*) of adjacent odontoblasts, which typically show only minute irregularities All micrographs of embryonic material included in this paper were made from sections of the growing zone of the mouse incisor ×10,000

FIGURES 2 and 3. Portions of the cell membranes (*CM*) of adjacent ameloblasts showing only slight irregularities (FIGURE 2, ×23,000) and pronounced folding (FIGURE 3, ×17,000)

duction of circumpulpal predentin begins. The latter stage is easily recognized, because the fibrillar basis has become diphasic. The Korff's fibers, now a minor component, pass as compact bundles of fibrils from between the odontoblasts through the circumpulpal predentin into the mantle predentin (FIGURE 9). The major fibrillar component of the circumpulpal predentin is also collagenous, but the fibrils are much smaller in diameter (350 to 500Å) and are distributed in a network. There is no visible anatomical association between the two groups of fibrils, and thus no evidence has been provided to support the idea

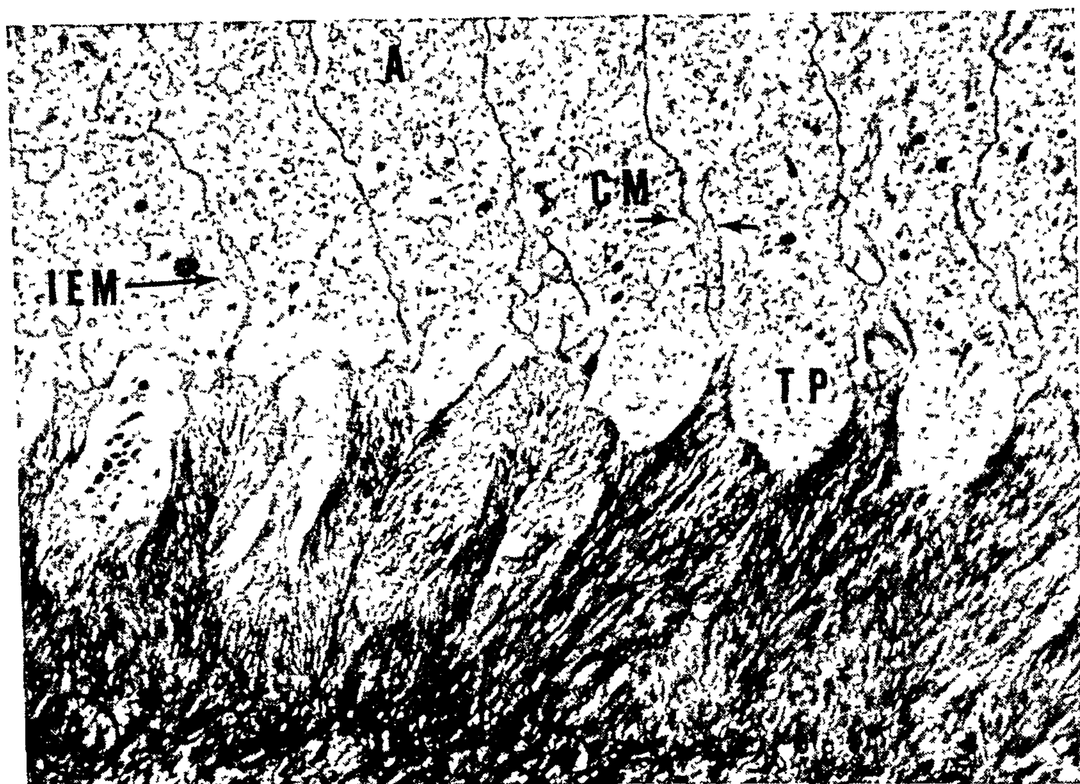


FIGURE 7 Row of accumulations of dense material (*IEM*) between the distal ends of the ameloblasts (*A*) Note distinct cell membranes (*CM*) on either side of deposits that appear identical to enamel matrix Together with the previously formed matrix, the intercellular material outlines the Tomes's processes (*TP*) ×12,600

proposed earlier that the smaller fibrils are derived from the Korff's fibers.[8] It has been observed, however, that near the ends of the odontoblasts the circumpulpal predentin is composed of a granular substance containing relatively small numbers of fibrils (FIGURE 10). On the other hand, in the more remote portion of the circumpulpal predentin, which is formed earlier, very little granular material is present, and the fibril content is tremendously increased (FIGURE 11). Taken in conjunction with the apparent increase of cytoplasmic activity within the odontoblasts, these observations may be interpreted as indicating that the elements that go into fibril formation emanate from the cells and that fibril synthesis occurs extracellularly *in situ*.

In the earliest description of amelogenesis as observed under the electron microscope it was reported that enamel matrix is produced as an amorphous

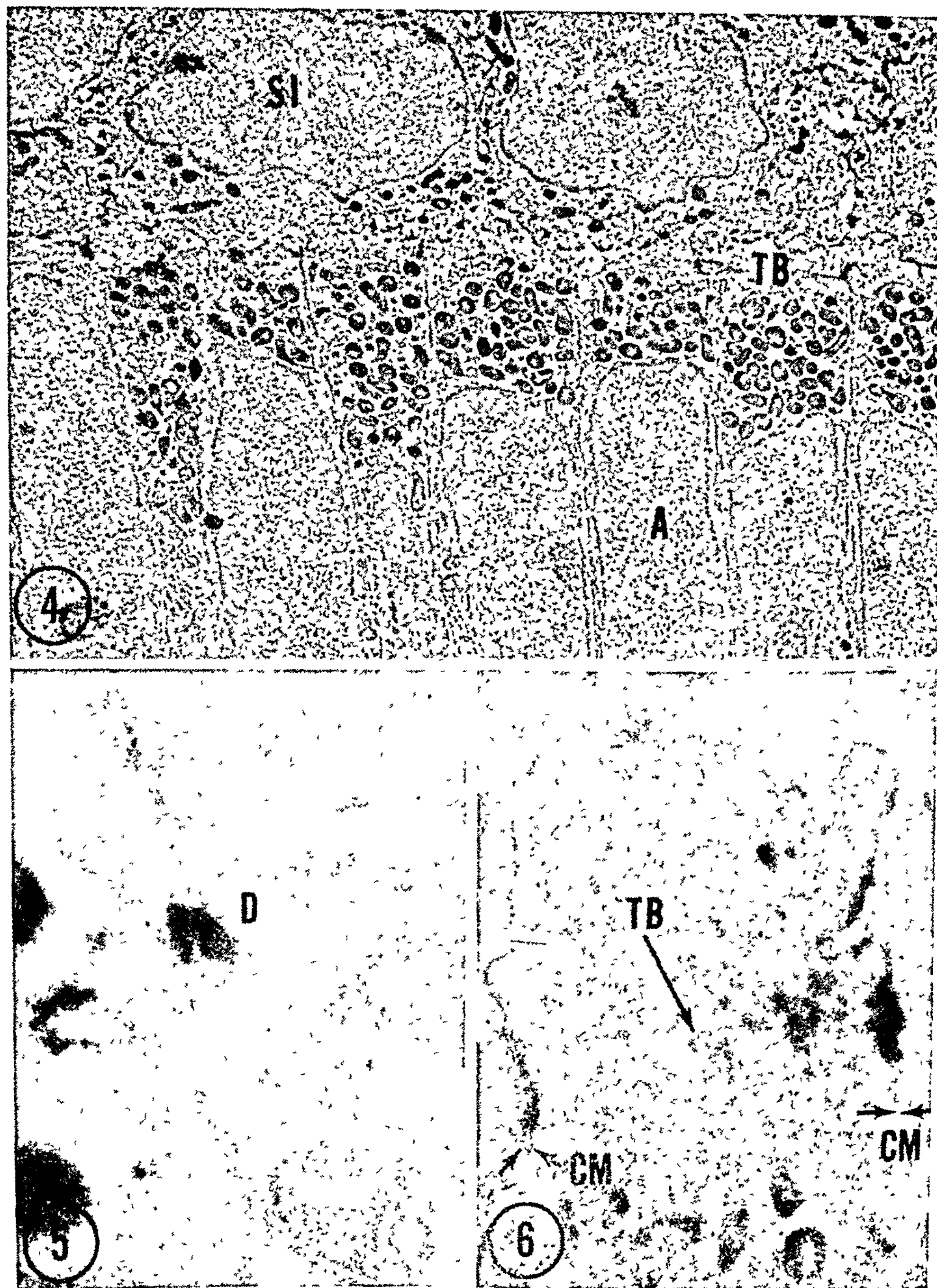


FIGURE 4 The terminal bar system (*TB*) at the basal ends of the ameloblasts (*A*). Cells of the stratum intermedium (*SI*) are at the top of the picture ×4600

FIGURE 5 A typical desmosome (*D*) Note the thickened cell membranes and increased density in the underlying cytoplasm of the ameloblasts ×50,000

FIGURE 6. A portion of the basal terminal bar system (see FIGURE 4) shown at higher magnification Note the band of dense cytoplasm (*TB*) extending across the cell and the thickened cell membranes at the ends of the band ×16 000

material that subsequently becomes fibrillar.[9] In later work, in which considerably thinner sections were examined, it was shown quite convincingly that fibrils are actually present from the onset of matrix formation.[6,10,11] In the latter studies, cell membranes were not observed at the distal ends of the ameloblasts. The conclusion was reached that intercellular matrix production does not occur as a distinct process, and thus that no component is produced that may be interpreted as interprismatic substance. In our most recent investigations we have been able to achieve further improvements in the preservation of the ameloblastic layer that have made it possible not only to observe cell membranes in the region of the Tomes's processes but also to demonstrate the intercellular deposition of matrix fibrils.[12,13] This has led us to the concept of enamel matrix formation that follows.

Prior to organized matrix production a thin continuous layer of fibrillar material is laid down along the dentinoenamel junction. This seems to be extracellular and appears to be connected with the future interprismatic substance. After this layer, which corresponds to the dentinoenamel membrane, is formed, small accumulations of dense material are deposited between cells, a few microns away (FIGURE 7). These are the structures that, as indicated in the foregoing, correspond to the optically designated terminal bars. The intercellular spaces distal to these deposits next become filled with matrix, which completely surrounds the ends of the ameloblasts, outlining the Tomes's processes (FIGURE 12). The cell membranes may be seen clearly along either side of the interprismatic matrix and around the Tomes's processes. At the basal tips of the interprismatic matrix projections the lateral cell membranes then reach across the ameloblasts and isolate the Tomes's processes. The bridges across the cells appear in sections as double membranes (FIGURE 13). The intermembranous space is continuous with the lateral intercellular spaces, and it becomes filled with material that is identical to and continuous with the intercellular matrix. The now isolated Tomes's processes are finally transformed, from the periphery inward, into fibrillar matrix (FIGURE 14). As the latter step proceeds, the first phase is repeated, new Tomes's processes being outlined basal to the preceding ones as a result of extracellular matrix formation.

If this interpretation of our findings should prove correct an explanation would be offered both for the "herringbone" configuration in rodent enamel and for the appearance of the periodic cross striations (rhythmic apposition lines) in fully developed human enamel prisms. The most recently published studies on human enamel, made with the light microscope, provide some additional support for the preceding ideas.[14]

The Structure of Mature Enamel and Dentin

The problems encountered in defining the cross-sectional contours of the prisms and in ascertaining the presence or absence of interprismatic substance in mature enamel have been described at length in previous publications.[15-17] Since almost no new information along these lines has been obtained, the present discussion will be limited to general observations on the mineral component of the enamel.

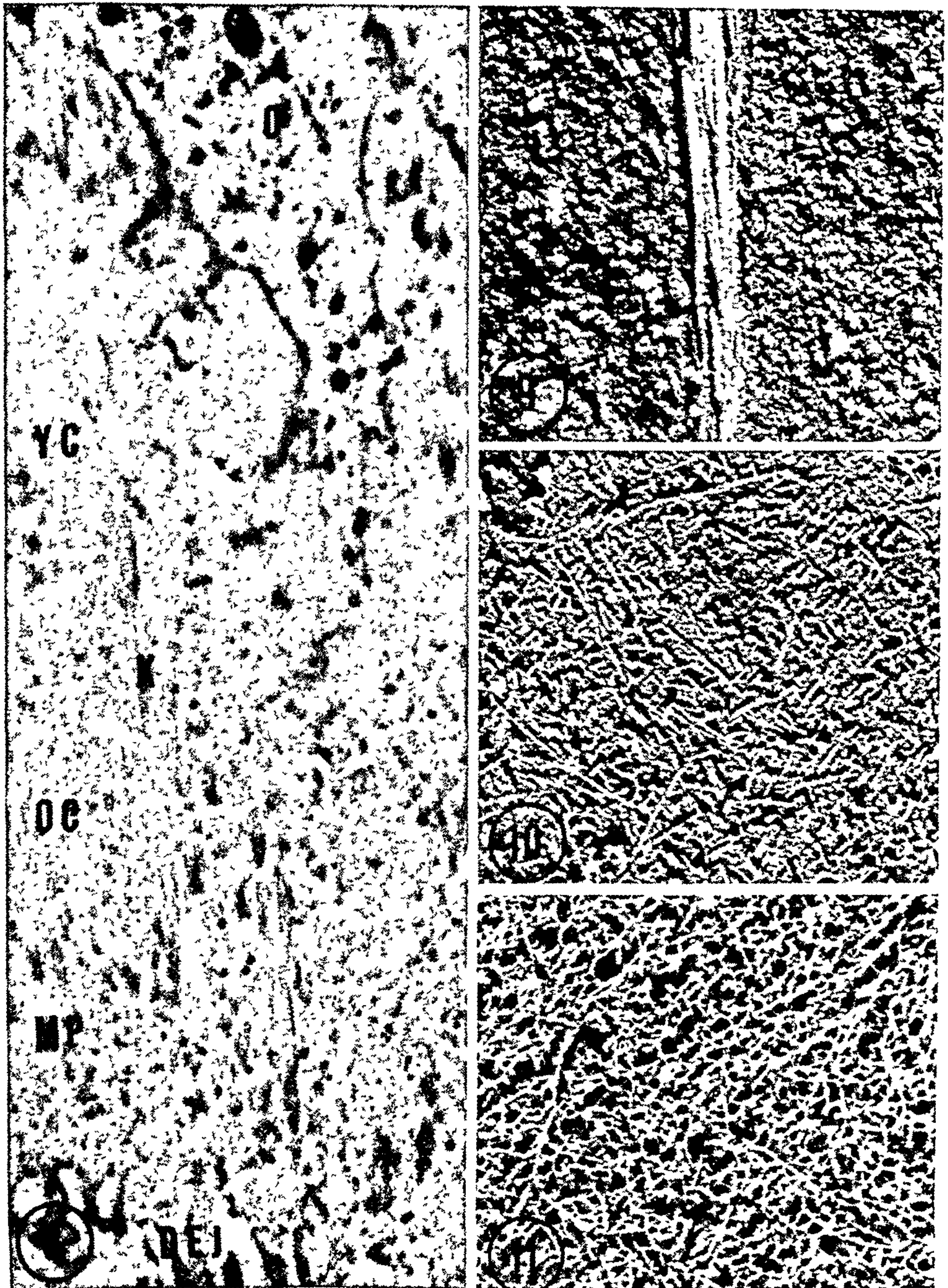


FIGURE 8. A section of developing predentin, showing the mantle (*MP*), old circumpulpal (*OC*), and young circumpulpal (*YC*) layers Korff's fibers (*K*) extend from between the odontoblasts (*O*) to the dentinoenamel junction (*DEJ*) ×10,000

FIGURES 9 to 11 Sections that have been shadowed after removal of the embedding medium in order to accentuate the Korff's fibers passing through the young circumpulpal predentin (FIGURE 9), the sparsely distributed fibrils in the young circumpulpal predentin (FIGURE 10), and the abundant fibrillar network in the old circumpulpal predentin (FIGURE 11). ×10,000.

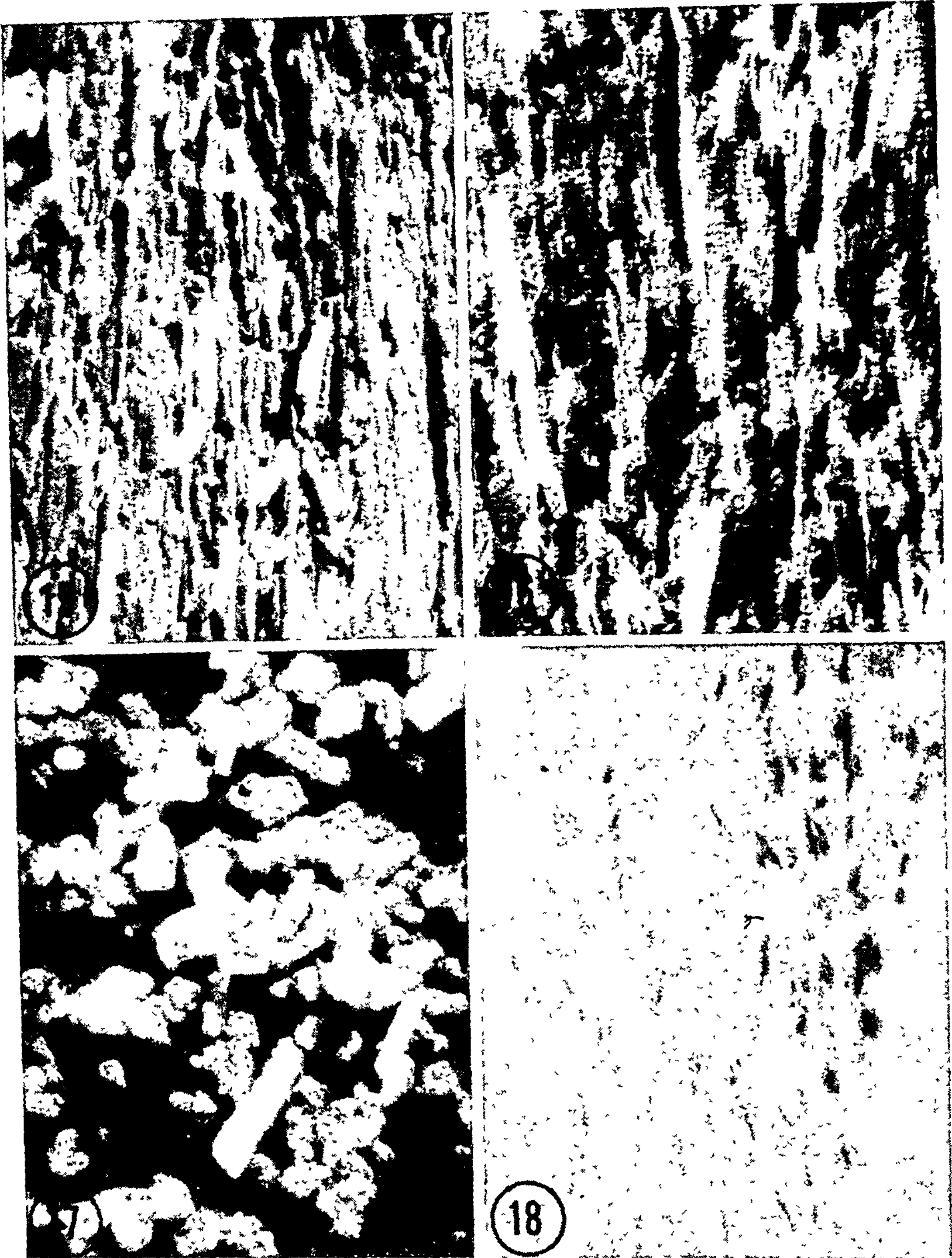


FIGURE 15. Marked variation in the lengths of the crystal-like objects in enamel, as seen in replicas of acid-etched longitudinal ground sections ×30,000.

FIGURE 16. Suggestions that the long crystal-like objects may be composed of smaller units, also seen in replicas of acid-etched ground sections ×30,000.

FIGURE 17. Small particles of crystalline material recovered on pseudoreplicas made from ground sections treated with organic solvents, in this instance ethylenediamine. ×65,000

FIGURE 18 Suggestions of alignment of the small individual crystals in very young developing enamel, as seen in sections. ×100,000.



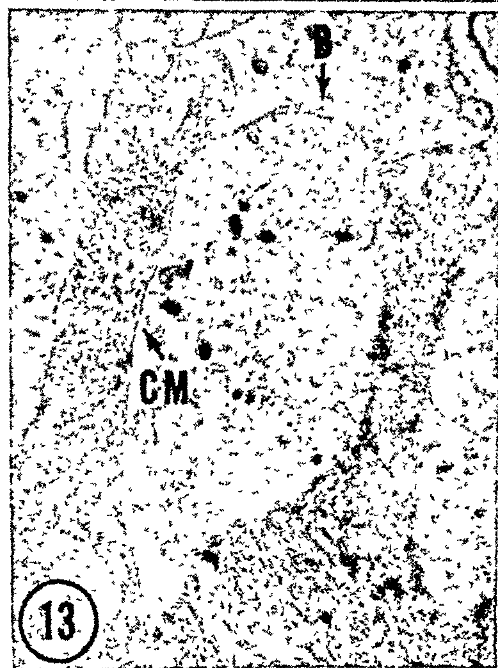




FIGURE 12 Isolation of Tomes's processes (*TP*) from ameloblasts and transformation of previously formed segments into enamel matrix ×6800

FIGURE 13. Isolated Tomes's process Double cell-membrane bridge (*B*) has formed across ameloblast and is partly filled with matrix Note continuity of bridge membranes with cell membranes (*CM*) and bridge matrix with intercellular matrix ×17,500

FIGURE 14. Advanced (*bottom*) and intermediate (*top*) stages in transformation of Tomes's processes into enamel matrix ×12,600

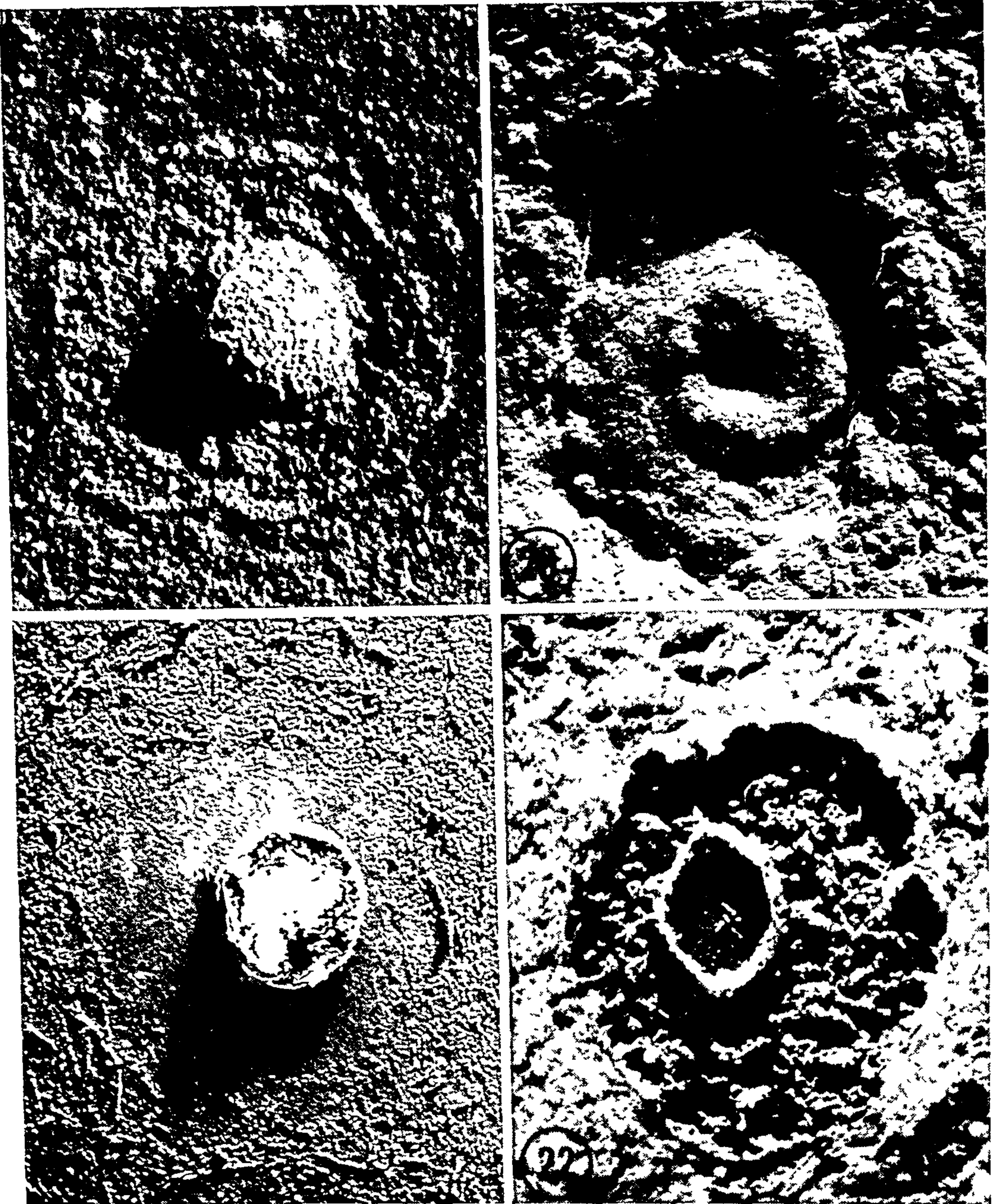


FIGURE 19 An early micrograph showing an apparently solid structure in the region around the odontoblastic process in mature human dentin. The preparation was a negative collodion replica of a lightly acid-etched ground cross section. ×20,000.

FIGURE 20 A later micrograph, made from a positive carbon replica of similarly etched dentin, in which the peritubular zone again appears solid ×20,000.

FIGURE 21. A recent micrograph, showing the relatively slight effect of an organic solvent, ethylenediamine, on the peritubular area, compared to the greater effect in the surrounding intertubular region The preparation was a negatively shadowed two-stage collodion-carbon replica. ×20,000.

FIGURE 22 The sparse organic matrix in the peritubular zone, as shown in a section of demineralized mature dentin

A wide range of measurements of the dimensions of the apatite crystals that constitute the major portion of the enamel has been reported The most striking differences occurred in the estimations of crystal length, which vary from 270 to 50,000 Å.[18,19] Although it is obvious that, especially in biological substances, the dimensions of the crystals that make up a solid need not be uniform, the available evidence does not warrant attributing this great variation simply to statistical distribution. The difficulties encountered in specimen preparation have made it difficult to state with certainty that the crystal-like objects observed were in fact single crystals and not aggregates of smaller units or fragments of larger ones (FIGURES 15 and 16).

The problem is complicated further by the fact that a fibrillar organic component is present also in fully calcified enamel. The interrelationship between the organic and inorganic phases is not clearly understood, and the possibility cannot be overlooked that the contours and configurations of the mineral crystals are strongly influenced by the matrix in which they are deposited[20] Furthermore, the actual presence of fibrillar material within crystal-like objects has been shown in micrographs made from sections of fully mineralized enamel.[18] In our work on mature enamel, carried out by means of pseudoreplicas, it has been found that the crystal-like objects are broken down into smaller fragments (FIGURE 17) as a result of treatment with such organic solvents as ethylenediamine or sodium thioglycollate.[19,21,22] These observations have led to the tentative idea that mineralization occurs at periodically spaced locations in the organic matrix, and that the growing particles come into contact with each other and are cemented into ribbonlike structures, the parallel arrangement and length of which are again determined by the configuration of the matrix A partial test of the validity of such a concept should be made possible by extending the work on developing enamel that is now under way. Preliminary studies in which electron microscopy has been supplemented with limited-area electron diffraction have already shown that, at the earliest stages of mineralization, single apatite crystals are in fact distributed in linear fashion within the organic matrix (FIGURE 18).

There are numerous open questions regarding the structure of mature dentin, and the latest information of interest concerns the constitution of the dentinal tubule. From the earliest days of electron microscopy the space between the odontoblastic process and the wall of the dentinal tubule has appeared to be filled with solid matter (FIGURES 19 and 20),[23,24] but the significance of this characteristic has not been brought into focus until recently Convincing evidence has now been derived by means of optical microscopy,[25] electron microscopy,[19,26-28] and microradiography[29-31] that supports the conclusion that the intratubular (also called peritubular) space is permeated by a sparse organic matrix and is mineralized to a greater degree than that of the surrounding intertubular substance (FIGURES 21 and 22). The phenomenon cannot be explained on the basis of secondary calcification, for it has been noted throughout preparations made from apparently normal dentin in the teeth of young persons, and even in unerupted teeth. Further information on this finding, which promises to exert considerable influence on current ideas regarding the physiological activity of dentin, will undoubtedly be forthcoming from the continuing studies of dentinogenesis that are in progress.

CRYSTALLOGRAPHIC STUDIES OF CALCIUM CARBONATE PHOSPHATE*

By Otto R. Trautz

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During the past century the dental tissues have been the object of most thorough studies with the microscope, and a great wealth of microscopic details has been collected. The explanation of many microscopic observations requires knowledge of structural details on a submicroscopic level Here the electron microscope allows us to discern morphologic details one hundred or even one thousand times smaller than those resolved by the light microscope. Crystallographic methods, on the other hand, furnish information regarding the submicroscopic structure of the building elements or their orientation.

Our chief crystallographic tools are X-ray diffraction and microscopy in polarized light. X-ray diffraction is most helpful in the study of crystalline materials, while from amorphous materials we can deduce little information beyond the fact that they are amorphous. The polarizing microscope is particularly useful in the study of birefringent materials. The birefringence is caused by certain atom groups, and it reveals their orientation when the groups are aligned more or less parallel. It does not matter whether the material is crystalline, but random orientation of the groups nullifies the effect.

This essay deals with crystallographic studies of calcium phosphates that contain appreciable amounts of calcium carbonate. I have been particularly interested in the manner in which the carbonate is included in the material. The materials have all formed from aqueous solutions. In the first section the X-ray diffraction of some *in vitro* precipitates, as well as some biological materials, is discussed. The second and third sections deal with the optical properties of the better crystallized mineral carbonate apatites and with what we can learn from them about the carbonate inclusions in these phosphates.

On the Carbonate-Containing Calcium Phosphates and the Phosphate-Containing Calcium Carbonates

With few exceptions the mineralized tissues of the animal world are impregnated with calcium phosphate or with calcium carbonate. In the vertebrates the crystallized mineral is a basic calcium phosphate. hydroxyl apatite; in the lower animals it is one of the various forms of calcium carbonate. calcite, aragonite, vaterite, or mixtures of them; or it may be carbonate in amorphous form The type of the crystalline material is easily identified by X-ray diffraction.

Chemical analyses reveal that the apatitic tissues always contain smaller or larger amounts of carbonate which, however, does not crystallize separately as carbonate We have never found in the diffraction patterns of native

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(see below). In curve *F* no indication of apatite or any other crystalline arrangement can be recognized. This is a truly amorphous material. An apatite precipitated from neutral solutions is always poorly crystallized, due to its very low solubility resulting in great supersaturation, and also to the preponderance of the HPO_4 ions in the solution which are coprecipitated in the apatite, substituting for some of the PO_4 ions. Material *C* is a poorly crystallized precipitate that must contain, in addition to the HPO_4, also some excess of OH, since the Ca:P molar ratio of the precipitate is that of the apatite (1.67). If this precipitate is heated for 20 hours in the autoclave at 360° C. and 200 at. steam pressure, the perfection of the crystals greatly improves through solution and recrystallization, as shown by curve *B*. Igniting this material in the muffle at 900° C. for 3 days permits further recrystallization by diffusion in the solid state, as shown by curve *A*, which is comparable to the curves obtained with well-crystallized mineral apatites.

The degree of disturbance is indicated by the lowering and broadening of the diffraction peaks. It is interesting to note that the reflections from the prismatic planes (*hk*0) and from the steep pyramid planes (*hkl*, with a high *hk*:*l*) are lowered much more than the reflections from the basal planes (00l) and from the flat pyramid planes (*hkl*, with a low *hk*:*l*). Thus, the (210), (300), (310) peaks are suppressed earlier, while the (002) and the (112) peaks remain longest. The broadening of the prismatic reflections may be interpreted as due to a distortion of these atomic planes, so that their spacings show appreciable fluctuations, and also to a reduction in the thickness of the needle-shaped crystals, so that fewer unit cells can diffract together as a coherent lattice.

The coprecipitation of carbonate causes an additional disturbance. It reduces the degree of crystallinity still further and enhances the irregularity in the aggregation of the ions, that is, the amorphous state. Materials *D*, *E*, and *F* have been precipitated at 37° C. and about neutral *p*H from phosphate solutions containing increasing amounts of carbonate. The phosphate:carbonate molar ratio for *D*, *E*, and *F* was 1:0 2, 1:2 and 1:20, respectively. Stirring of the precipitates in their solutions was continued for half an hour to make certain that the amorphous state is not just a momentary one. While in curve *E* the (002) spacing (3 44 Å) of the apatite is still recognizable, it has completely disappeared from curve *F*. Such material is no longer an apatite. It is amorphous Simultaneously, the flat maximum of the broad band has been extended downward to 29°.

The difference between a very poorly crystallized apatite and a completely amorphous material can also be recognized on X-ray diffractograms obtained in the microcamera. FIGURES 2, *E* and *F*, were obtained with the same materials which gave curves *E* and *F* of FIGURE 1. The presence of the (002) ring of the apatite is quite evident on *E*. This photographic test for the (002) ring is very sensitive and can be performed on very small samples, such as sections of biological materials, 0.1 mm. in diameter.

The minerals in the carbonate-impregnated tissues often are very pure calcium or calcium-magnesium carbonates. They are very well crystallized, in large grains, giving sharp diffraction lines (for example, shells of many mollusks).

biological materials lines of crystallized carbonate together with those of the apatite.

The carbonate disturbs the crystallization of the apatite: the crystals are smaller and more distorted in the dentine and bone with the higher carbonate

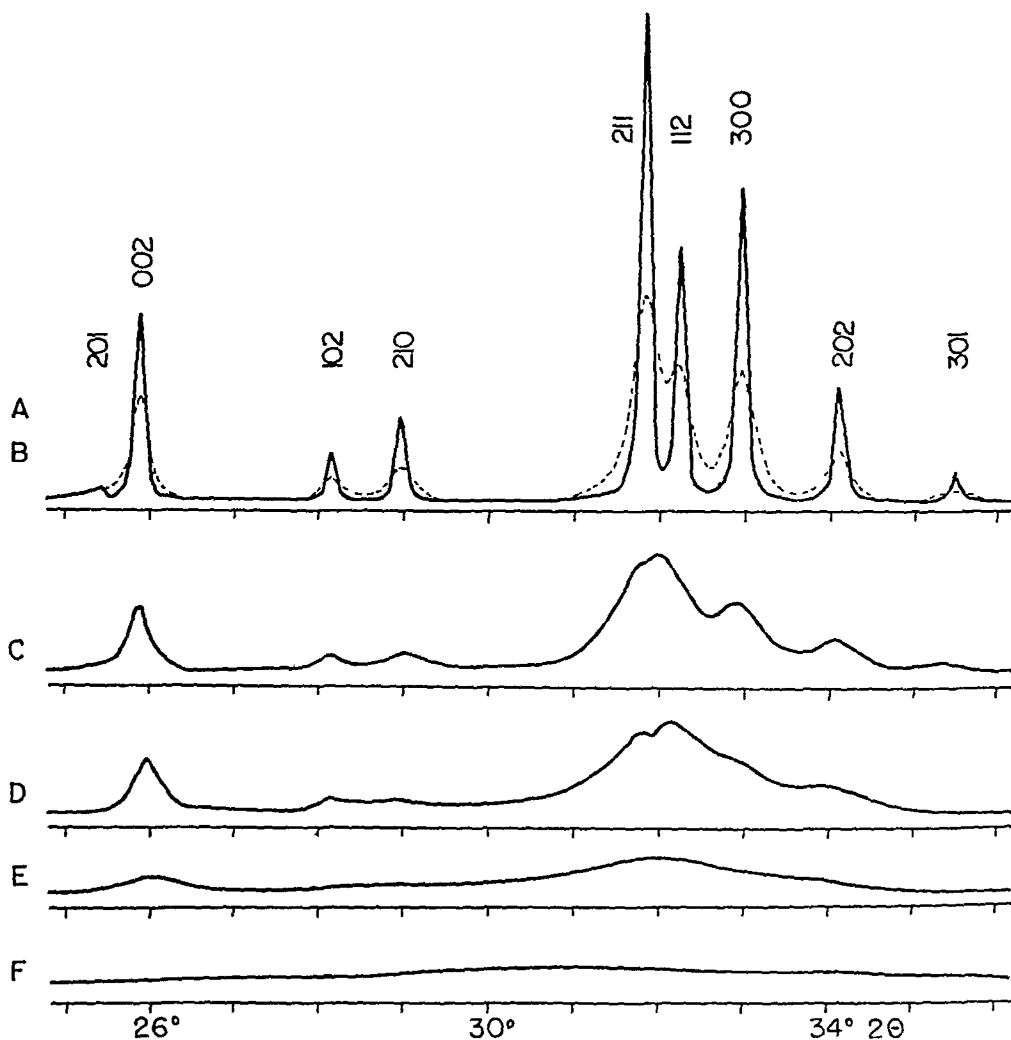


FIGURE 1 Diffraction curves of calcium phosphate precipitates obtained in a Norelco diffractometer with CuKα radiation (diffracted intensity versus diffraction angle 2θ) The peaks are named by the Miller indices of the reflecting planes in the apatite crystals The degree of crystallization of the poorly crystallized precipitate *C* is improved by autoclaving (dashed curve *B*) and by ignition (curve *A*). Presence of carbonate in the solution reduces the crystallinity of the precipitate further (curves *D* and *E*) and eventually causes an amorphous precipitate to form (curve *F*)

content than in enamel. Likewise, *in vitro* precipitations of calcium phosphate from solutions with increasing amounts of carbonate yield increasingly disturbed apatite precipitates.

FIGURE 1 shows the diffraction curves of various calcium phosphate precipitates. Curve *A* is that of a well-crystallized hydroxylapatite powder. The curves *B* to *E* come from samples of increasingly poorer crystal quality, although the apatite arrangement of the ions in the crystals is still indicated

However, there are other tissues that contain appreciable amounts of phosphate mixed with the carbonate. The phosphate disturbs the crystallization of the carbonate more than the carbonate disturbs the crystallization of the phosphate. As little as 5 mole per cent phosphate present in the carbonate solution can give a precipitate which is completely amorphous (FIGURE 1F). It is an unorganized heap of calcium, phosphate, and carbonate ions.

I have found such amorphous mineral deposits in a number of biological materials, in the principal layer of the lobster carapace, in the calcified tendon of the lobster claw, and in tendons inside the frontal region of the stone crab and others.

In the segments of the tapeworms (*Cysticercus* sp.: and *Taenia* sp.:) egg-shaped calcareous granules* of about 5 μ diameter are found that can be isolated from the tissue by heating it for 6 hours in ethylenediamine at 100° C. The granules consist of calcium and magnesium carbonate and contain 5 wt. per cent P. Accordingly, about 25 per cent of the calcium is bound to phosphate.

These amorphous materials give diffractograms such as that in FIGURE 2F. For our investigations it is of great importance to know that the treatment with ethylenediamine (85 per cent) does not cause a recrystallization of the originally amorphous carbonate-phosphate. Treatment with 60 per cent aqueous KOH definitely causes a recrystallization of the originally amorphous material, probably accompanied by a change in composition.

Prenant[1] has already reported that the calcium carbonate deposits in tissues are found to be amorphous when their molar $PO_4:CO_3$ ratio is 0.1 and higher, and that the presence of magnesium carbonate extends the range of the amorphous phase. If, however, a CO_3 enrichment from the core toward the surface of the crystal is found, it can be postulated that the $CO_3:PO_4$ ratio in the solution must have changed during crystallization. When, for example, the phosphate supply is limited and that of calcium and carbonate is not, the outer layers of the crystals formed may become progressively richer in carbonate.

I consider the carbonate as being coprecipitated with the phosphate as an adventitious impurity since, like the phosphate, it forms a very poorly dissociating bond with the calcium ion. Any other anion forming a poorly dissociating bond with calcium (for example, citrate) would be likewise coprecipitated. It is because of this bond that the carbonate is included in the precipitate and not because the apatite structure is more stable with the carbonate than without it. In my investigations I have not observed any indication for the existence of a double salt of calcium carbonate and calcium phosphate that would be more stable and would contain the carbonate and phosphate in a stoichiometric ratio and would thus give a characteristic diffraction pattern of its own. Instead, I found only that the apatite and calcite structures as the end members of the binary series are increasingly disturbed by the admixture of the other component, resulting in an amorphous phase in the middle of the series.

* Theodore C. von Brand at the National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Md., has isolated and analyzed the material and has kindly sent me a sample for examination by X-ray diffraction.



FIGURE 2. X-ray diffractograms, obtained in the microcamera *E*, very poorly crystallized apatite; *F*, amorphous carbonate phosphate precipitate The materials are the same as those used for FIGURE 1, *E* and *F*.

directly whether the carbonate is within the apatite structure. Only changes in lattice dimensions that accompany the substitutions could tell us. In first approximation we assume that the dimensional changes due to several simultaneous substitutions are additive. In the mineral carbonate apatites we usually find the *a*-axis shorter than in the carbonate-free apatites. The difference is roughly 0 01 Å per weight per cent CO_2.[4] Accordingly, we expect the human enamel apatite to have an *a*-axis roughly 0.02 Å shorter than pure OH apatite. Instead, we find it to be 0.02 Å longer, namely 9.441 ± 0.006 Å, as compared with 9.421 ± 0.003 Å for our standard synthetic OH apatite.[4] Of the +0.04 Å difference we might ascribe 0.01 Å to the effect of 0.03 wt. per cent Cl in the enamel, substituting for OH in the apatite. The cause of the remaining 0.03 Å expansion has not yet been definitely explained. Perhaps the presence of some coprecipitated HPO_4 (that is, when the molar ratio Ca/P in the enamel is below 1.67) could have an expanding effect on the *a*-axis of the apatite, as previously (Trautz,[4] p. 704) indicated. I have no useful data on a dimensional effect of coprecipitated citrate, because some precipitates that I studied were too poorly crystallized to be helpful. Similarly, in dentine and bone the apatite is too poorly crystallized to give a helpful diffraction pattern.

Whether there is some amorphous phase present in enamel cannot yet be decided. To derive the answer from X-ray diffraction curves would require accurate counting procedures of line and background intensities at various regions of the curves, and the comparison with a curve constructed by the theoretical extrapolation of the "tails" of the diffraction peaks. On the other hand, we know that after eruption the enamel still picks up additional mineral and becomes more highly mineralized. This pickup is fastest right after eruption and slows down with age as the spaces between the crystals are filled in and the diffusion channels become more restricted. This additional material causes the apatite crystals already present to become thicker or deposits itself otherwise between the crystals, displacing water in the swollen matrix. It probably is rather of the carbonate-poor better-crystallizing type than of the carbonate-rich amorphous type.

On the Structure of the Mineral Carbonate Apatites

The crystal structure of the apatite was established thirty years ago by Náray-Szabó[5] and by Mehmel.[6] A refinement of the atomic position in it has been reported by Posner *et al.*[7] A stereoscopic picture of a model of the structure is presented in FIGURE 3. If the carbonate is, as we believe, located within the apatite structure, then our problem is how to fit it into the structure. There are no interstices large enough to accommodate the large CO_3 group. Thus, the possibility of an interstitial solid solution is ruled out, and there remains only that of a substitutional solid solution. Again, the CO_3 group is too large to take the place of the OH ions on the hexagonal axis There remains, as the only possibility, the substitution for a PO_4 group, in spite of the difference in shape and electric charges. A carbonate ion temporarily adsorbed to the surface of a growing crystal is usually pushed away by a phosphate ion that fits better into the lattice space. However, sometimes it hap-

When, during the growth of an apatite crystal, a carbonate ion happens to diffuse from the solution to the surface of the crystal, it is held there by a group of calcium ions, as would a phosphate ion, with which it competes. The binding of phosphate in the apatite structure is stronger than that of carbonate, so that the crystal discriminates against the inclusion of the carbonate. As a result, the CO_3:PO_4 concentration ratio in the crystal is lower than the CO_3:PO_4 ratio in the solution from which the crystal grows or with which the crystal is in equilibrium. A quick precipitation (nonequilibrium) by a rapid addition of calcium ions would tend to diminish the discrimination and to raise the CO_3:PO_4 ratio in the crystal toward that of the solution. As long as the composition of the surrounding solution remains constant, we expect the composition of the growing crystal also to remain constant Thus, as long as the tissue fluids from which the apatite crystallizes stands in constant exchange with the surrounding tissues, keeping its CO_3:PO_4 ratio constant, we do not expect the carbonate to be lacking inside the crystals and concentrated on the surface. The presence of amorphous mineral in the principal layer of the lobster is of interest, as it is partly resorbed before the integument is shed in the regular moulting cycle[2] The amorphous state doubtless facilitates the remobilization of the mineral in this tissue.

A carbonate ion cannot exchange places in the crystal by diffusion. Any such exchange must take place by dissolution and new crystallization. A quickly precipitated material with a comparably higher CO_3 content is less stable and can recrystallize by way of solution to a material containing the lower equilibrium concentration of CO_3. Similarly, when the CO_3:PO_4 ratio of the surrounding solution is lowered, such recrystallization will take place, although perhaps only in the surface layers. This, we think, explains the observation of Brudevold *et al.* elsewhere in these pages that the CO_3 content of the outer enamel layers is appreciably lower than that of deeper layers. Recrystallization of the surface layers in contact with the saliva has resulted in a loss of CO_3.

In a recent investigation Fosdick[3] (also personal communication) has mechanically comminuted enamel and separated it into light and heavy fractions. The light fraction containing a higher concentration of organic material (2.3 wt. per cent) supposedly comprises the matrix-rich portions of the prism cortices, while the heavy fraction comprises the more highly mineralized prism cores (0.27 per cent organic). In Fosdick's analyses the light fractions yielded, surprisingly, a lower carbonate value (2.35 wt. per cent) than the heavy fractions (3.02 wt. per cent). It is known that water and dissolved salts and acids can diffuse through the enamel in both directions. I believe that the matrix fibers in the enamel serve as the diffusion channels, and I find it very likely that along these channels a fractionated solution of the accessible carbonate occurs together with a recrystallization of the remaining phosphate with a reduced carbonate content. Another possibility should be investigated, namely, whether in the latter stages of the enamel "maturation," after eruption of the tooth, the material precipitated from the saliva in and on the surface of the enamel has a lower CO_3:PO_4 ratio than the original enamel material.

From the X-ray diffraction patterns of human enamel, we cannot learn

pens that the carbonate remains attached and the crystal continues to grow around it, while the carbonate group arranges itself in the space left vacant by the displaced PO_4 group.

The unit cell of the apatite contains one formula weight of $Ca_{10}(PO_4)_6(OH)_2$. If we assume that all of the carbonate present in the apatite is part of the structure, the presence of 2 wt. per cent CO_2 in the apatite, as in enamel, would mean that, on the average, in almost every second unit cell one PO_4 group is replaced by a CO_3 group. The CO_3 will be randomly distributed throughout the structure. The force field of the CO_3 groups is different from that surrounding the PO_4 group, and it causes a distortion of the atomic planes and, at times, a break in the coherence of the lattice, as evidenced by the broadening of the diffraction lines.

The biological apatites are not sufficiently well crystallized to give us any further clue as to the positions of the carbonate in the apatite. The mineral apatites are usually better crystallized. There are large deposits of phosphorite rock, which consist of microcrystalline carbonate fluor apatites of marine origin. However, well-developed single crystals of carbonate fluor apatite (francolite) and carbonate hydroxyl apatite (dahllite) are found in comparatively few places From these crystals we can obtain more helpful information on the structure of the carbonate apatite.

The diffraction lines even of these single crystals are not as sharp as those of the usually well-crystallized fluor apatites, illustrating the above-mentioned lattice distortions. All the mineral carbonate-containing apatites have shorter *a*-axes and somewhat longer *c*-axes than the corresponding carbonate-free F and OH apatites. For this reason we assume that the carbonate is located within the apatite structure. We could describe the carbonate apatite as an anomalous (not isomorphous) mixed crystal of $CaCO_3$ and apatite. This is as far as X-ray diffraction can help us.

Fortunately, there is another crystallographic tool, microscopy in polarized light, that can lead us a step further in positioning the carbonate groups in the apatite. The strong directional effect of the carbonate groups on light manifests itself in the birefringence, which is often observed in carbonate-bearing crystals. The tetrahedral PO_4 groups have optically an almost spherical symmetry, and they impart almost no birefringence to the apatite, whose birefringence therefore is very low ($n_e - n_o = -0.003$). On the other hand, however, the planar triangular CO_3 groups can impart a very strong birefringence to a crystal, due to the great difference in polarizability of the carbonate group in the direction of the plane of the group and normal to it.

Light can interact with the carbonate group, or "polarize" it, much more strongly when the vibration direction, that is the direction of the electric vector is in the plane of the group than when it is normal to the plane: the polarizability of the CO_3 group in the direction of the plane (β) is greater than the polarizability in the direction normal to the plane (α). When the CO_3 groups in a crystal are all oriented parallel, say, normal to the *c*-axis, then the principal polarizability of the crystal, μ_x (and the refractive index n_o) for vibrations perpendicular to the axis is greater than the polarizability, μ_z (and the refractive index n_e) for vibrations parallel to the *c*-axis. The difference in the principal polarizabilities of the crystal (or "bipolarizability," as we may

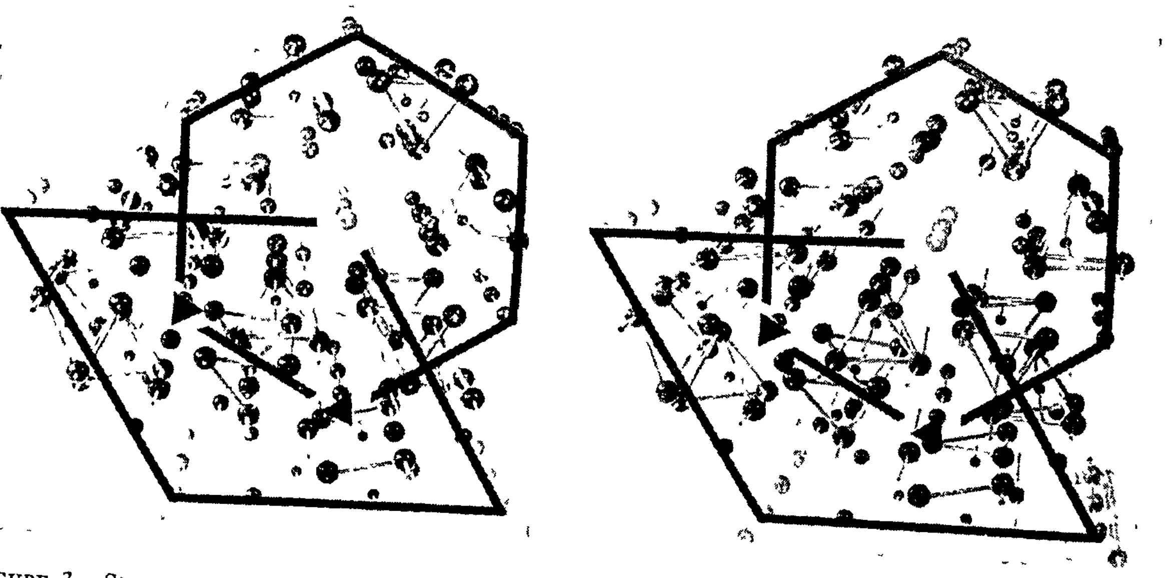


FIGURE 3 Stereoscopic view of a model of the apatite structure, looking parallel to the hexagonal axis down on the basal plane (this model was built by Edward Klein). The hexagonal unit cell of the structure is outlined by the taped rhombus, with the *c*-axes at its corners. It contains 10 Ca (single dark spheres), 6 PO_4 (tetrahedra), 2 OH or F 9 (single light spheres on the *c*-axes) The depth of the unit cell extends from the first to the third OH The taped hexagon brings into view the hexagonal symmetry of 1 of the 4 *c*-axes, 2 of which belong to the unit cell outlined. The PO_4 tetrahedra have 1 vertical and 1 horizontal edge and 2 vertical and 2 tilted faces There are 2 symmetry planes perpendicular to the *c*-axes They pass through the horizontal edges of the PO_4 tetrahedra and also through the OH and the Ca ions not lying on the trigonal axes

With a little practice one may succeed, without the use of a stereoscope, in seeing the stereoscopic picture in space Place the 2 well-illuminated pictures symmetrically before you Align your eyes parallel by viewing "through the pictures" an imaginary distant spot While the eyes accommodate to the closer distance of the pictures (25 to 30 cm) the 2 pictures fuse into a single 3-dimensional view A piece of cardboard placed vertically between the 2 pictures may be of help at the beginning

the dahllite with that in calcite: $P_{\text{dahllite}}/P_{\text{calcite}} = -0.632/-0.204 = 0.309$. This means that the observed bipolarizability contribution of the CO_3 groups in dahllite is only 31 per cent of that in calcite, where all CO_3 groups are in horizontal position.

We may interpret this difference by assuming that only 31 per cent of the CO_3 groups present in the dahllite are oriented horizontally, while the other 69 per cent are randomly oriented, as in an amorphous phase, where we would not expect an appreciable orientation of these groups.

Another probable interpretation is that the carbonate groups in the dahllite are tilted out of the horizontal plane, thus reducing the contribution of each group to the bipolarizability of the dahllite to 31 per cent of its maximum As the horizontal groups are tilted up toward a vertical position and the tilt angle increases from 0 to 90°, the negative bipolarizability and birefringence contribution decreases and passes through zero and reverses to positive. We now want to know in what measure the contribution varies with the tilt angle of the groups. We plot the relation in a curve and then read from the curve the

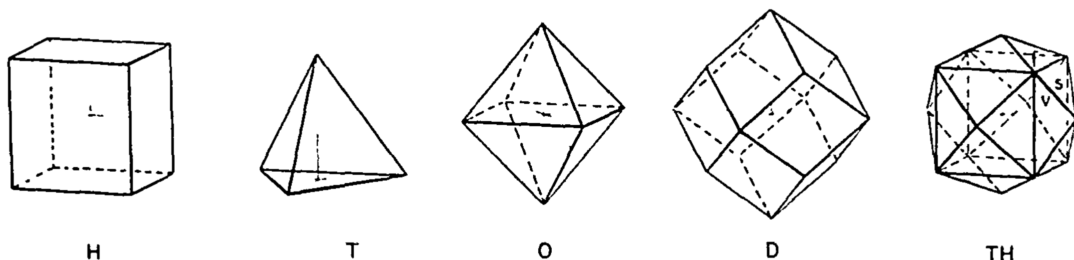


FIGURE 4 Various polyhedra of the cubic system hexahedron *H*, tetrahedron *T*, octahedron *O*, dodecahedron *D*, tetrahexahedron *TH*

tilt angle of the groups at which their contribution is only 31 per cent of the maximum.

For the construction of this curve we obtain a few fixed points by a simple crystallographic consideration: a cubic crystal is always isotropic. If there is one atom grouping in it which causes birefringence by the symmetry conditions of the cubic system, the group must be repeated in such a way that the birefringence contributions of the several groups neutralize each other. As an example, a CO_3 group lying in one cube face (FIGURE 4) is repeated in all faces of the cube to conform to the isotropic property of the cubic system. The birefringence contribution (with regard to the vertical axis) of the one horizontal CO_3 group (tilt angle $\tau = 0°$) is taken as one negative unit ($B_0 = -1$). Then the contribution of the 2 horizontal groups ($2B_0 = -2$) is neutralized by the contributions of the 4 vertical groups ($4B_{90} = +2$). Thus, at a tilt angle $\tau = 90°$, the birefringence contribution per group, B_{90}, amounts to $+0.5$.

In an analogous way, we may visualize the other polyhedra of the cubic system (FIGURE 4) and obtain further points of the curve. In the tetrahedron, for instance, one horizontal group ($B_0 = -1$) is neutralized by 3 groups in the threefold pyramid, which have a tilt angle of 70 5°. Thus $B_{70\ 5} = +0.333$. In the fourfold pyramid of the octahedron with a tilt angle of 54 7° the vertical and horizontal components balance each other: $B_{54\ 7}$ = zero. In the do-

call this difference in analogy to "birefringence"), $\mu_z - \mu_x$, is proportional to the concentration of the carbonate groups, M, and to the difference in the polarizabilities of the groups, P, which depends on their orientation with regard to the c-axis and its normal plane. The bipolarizability of the crystal, $\mu_z - \mu_x$, on the other hand, is proportional to its birefringence, $n_e - n_o$, or in a more precise form, is equal to

$$\frac{n_e^2 - 1}{\frac{1}{3}(n_e^2 + 2)} - \frac{n_o^2 - 1}{\frac{1}{3}(n_o^2 + 2)}$$

When all CO_3 groups are oriented normal to the vertical c-axis the birefringence has a negative sign and is maximal. When the carbonate groups are tilted out of the horizontal plane, their birefringence effect becomes weaker and, when they are randomly oriented, their birefringence effect must be zero. Examination of the crystal in polarized light and the determination of the birefringence may give us some information on the orientation of the carbonate groups.

All carbonate apatites have a higher negative birefringence than the corresponding carbonate-free apatites. One naturally correlates this increased birefringence with the presence of the carbonate groups and with their orientation. In the following I try to acquire an idea of their orientation in the carbonate apatites by comparing the birefringence of the carbonate apatite with that of calcite in which, as we know, all carbonate groups are horizontal, that is, normal to the c-axis. Calcite thus exhibits a high negative birefringence. I shall in first approximation neglect the minor influences of the neighboring atoms and assume that the birefrigence of the calcite is due solely to the polarizabilities of the carbonate groups

The CO_2 content of calcite (44.0 wt. per cent) and the density (2 71 gm /ml.) give us the carbonate concentration in calcite M_c = 1.192 gm CO_2/ml The refractive indices are n_o = 1.658 and n_e = 1 486 Thus, the bipolarizability of calcite, $(\mu_z - \mu_x)_c$ is 0.862 − 1.1055 = − 0 2435 = M_cP_c, and division by M_c gives the bipolarizability contribution per gm. CO_2/ml.·

$$P_c = -0\ 2435/1.192 = -0\ 204 \text{ per gm. } CO_2/\text{ml}$$

Since all CO_3 groups are oriented horizontally, their contribution to the bipolarizability and to the birefringence of the calcite is maximal.

Geiger[8] has compiled values for the CO_2 content, density and birefringence of various carbonate apatites. The birefringence increases roughly proportional to the CO_2 content From Geiger's Figures 2 and 4 we take as representative values those of a dahllite from Greenland: 8.6 wt. per cent CO_2, density = 3.04 gm./ml, n_o = 1.615, n_e = 1 600, and correct for the slight birefringence (−0.003) of the carbonate-free apatites by reducing n_o to 1.612, while 0.012 is the increase in the birefringence of the apatite due to the presence of the carbonate. M_d amounts to 0 2614, and the bipolarizability of dahllite is 1 0265 − 1.043 = −0.0165 = M_dP_d or

$$P_d = -0.0165/0.2614 = -0.0632 \text{ per gm. } CO_2/\text{ml.}$$

We now can compare the bipolarizability contribution per gm. CO_2/ml. in

For a complete calculation of the curve* one arranges N CO_3 groups in the faces of an N-fold pyramid about the vertical axis, thus preserving the uniaxial symmetry. The difference in the principal polarizabilities of this structure, $(\mu_z - \mu_x)$, is connected with the intrinsic polarizabilities of the CO_3 groups, $(\beta - \alpha)$, and with the refractive indices, n_o and n_e, by the following equation:

$$\mu_z - \mu_x = N(\beta - \alpha)B = \frac{n_e^2 - 1}{\frac{1}{3}(n_e^2 + 2)} - \frac{n_o^2 - 1}{\frac{1}{3}(n_o^2 + 2)}$$

in which N denotes the number of CO_3 groups in the pyramidal structure and B denotes the contribution of one CO_3 group to the difference in the polarizabilities of the structure. B is a function of the tilt angle τ between the plane of the group and the horizontal: $B = \frac{3}{2}\sin^2\tau - 1$; $\mu_z - \mu_x$ is maximal when all groups are horizontal, with $\tau = 0°$ and $B = -1$. The calculated B values for various tilt angles are listed in TABLE 1 and are plotted in the curve of FIGURE 5 The unit of B is $(\beta - \alpha)$.

TABLE 1

τ	B	τ	B
90	+0.5000	0	−1.000
85	+0 4886	5	−0.9886
80	+0 4548	10	−0.9548
70	+0.3245	20	−0.8245
60	+0.1250	30	−0.6250
50	−0.1198	40	−0 3802
45	−0 2500		

We return now to our original problem and read from the curve in FIGURE 5 that the bipolarizability contribution of the CO_3 groups is reduced to 31 per cent of its maximum value at a tilt angle of 43°. We obtain a similar value (tilt angle 41°) if we compare the dahllite with aragonite instead of with calcite, though aragonite has a 10 per cent higher density and an 11 per cent lower birefringence. This, then, is the approximate tilt angle if all CO_3 groups have the same inclination. One could also consider that the inclination of the groups may be distributed over a range of tilt angles, so that the over-all B contribution of the groups will remain 31 per cent of the maximum.

We now can support with quantitative data our objection[4] to McConnell's[9,10] arrangement of the CO_3 groups in francolite (1 horizontal and 3 vertical around the threefold axis), which cannot be correct, as it should produce in our dahllite a birefringence that is about 0.005 more positive instead of the observed 0.012 more negative than that of the carbonate-free apatite (−0.003). In the case of McConnell's model, apatite with high carbonate contents should exhibit a weakly positive birefringence, rather than a generally observed increased negative one. Two of the vertical groups neutralize the horizontal group, leaving 1 vertical group of 4 contributing the positive effect.

* I am indebted to P. P Ewald, Polytechnic Institute of Brooklyn, Brooklyn, N Y, for the derivation of this relationship.

decahedron, 4 vertical groups are neutralized by 8 pyramid groups at a tilt angle of 45°: $B_{45} = -0.25$. In the tetrahexahedrons, eight groups on the vertical faces, *v*, are neutralized by 16 groups on the two fourfold flat pyramids,

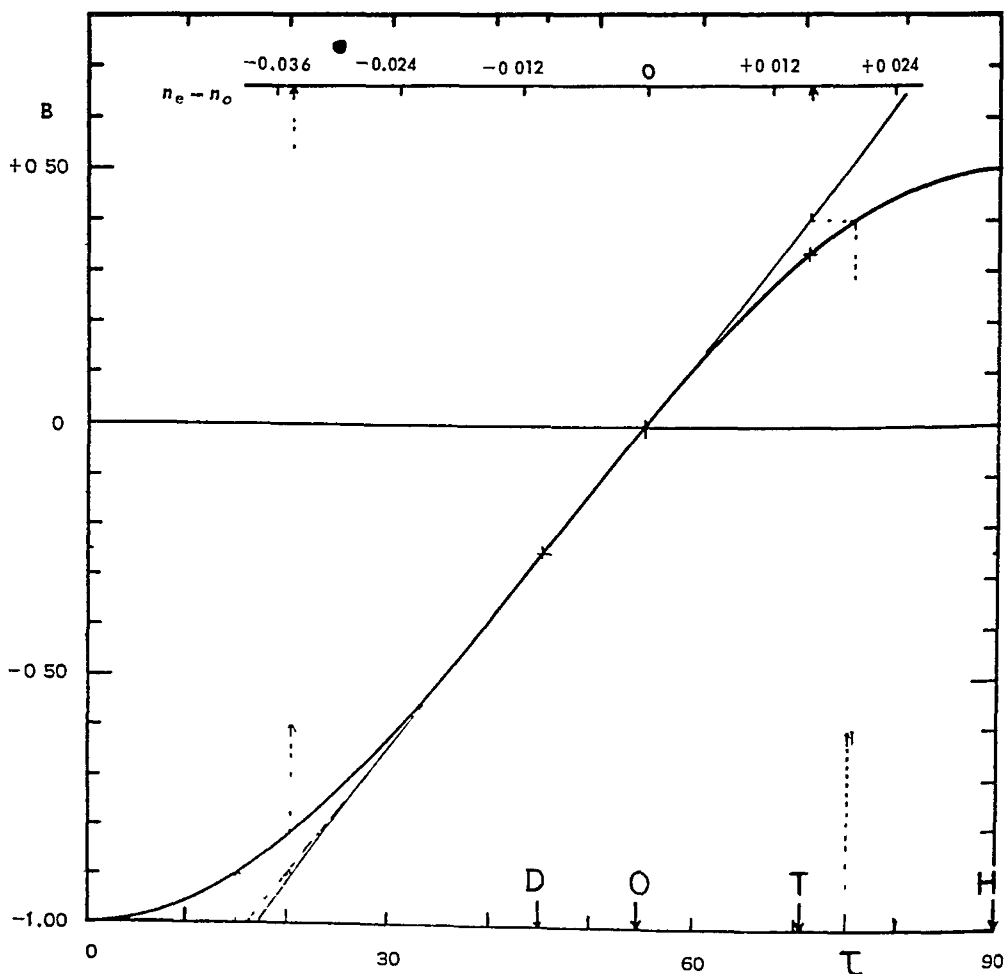


FIGURE 5 The *S* curve shows the contribution *B*, per CO_3 group, to the difference in the polarizabilities of the structure, as a function of the tilt angle τ of the group $B = \frac{3}{2} \sin^2 \tau - 1$ *D*, *O*, *T*, and *H* indicate the tilt angles of the pyramid faces of the dodecahedron, octahedron, tetrahedron, and hexahedron, respectively The upper scale shows the birefringence ($n_e - n_o$) which would be expected in our dahllite crystal when all its CO_3 groups were tilted at the angle τ (follow the dotted guide lines, and add −0 0003). The diagonal straight line demonstrates the good proportionality between the birefringence and *B* within a wide range of the tilt angles.

f, and on the 2 fourfold steep pyramids, *s*. The tilt angles of *s* and *f*, τ_s and τ_f, depend on the particular indices of the faces (120, 130, 230, and so on), but τ_f is always $90° - \tau_s$. From this consideration we can deduce that the curve $B = f(\tau)$ must have an S shape and must be symmetrical about its midpoint at $\tau = 45°$ and $B = -0.25$. The sum of $1B_f$ and $1B_s$ is then always equal to -0.50. We now have 7 points along the curve and can interpolate it with sufficient precision (FIGURE 5).

taneously. If (with crossed nicols and gypsum plate inserted) at a certain position they appear blue, then, after 60° and 120° rotation, the next and third pair of sectors will be blue. The border between 2 adjacent sectors, for example (100) and (010), runs from the uniaxial core to the corner of the hexagon. It is not always a straight line, but may be wavy and does not always run the same course in the higher and lower levels of the thin section so that, at times, layers of one sector overlap with layers of the other sector, resulting in an apparent extinction direction between the layers of the 2 main sectors.

In the carbonate-free apatites this optical anomaly is not observed. Thus we naturally attribute it to the presence of the carbonate groups. A concentration gradient in the composition of the crystal (for example, carbonate content) between core and surface cannot explain this optical behavior. Evidently the surface of the growing crystal is involved in producing this phenomenon, for example, by influencing the orientation of the carbonate groups while they are adsorbed to and included in the growing apatite.

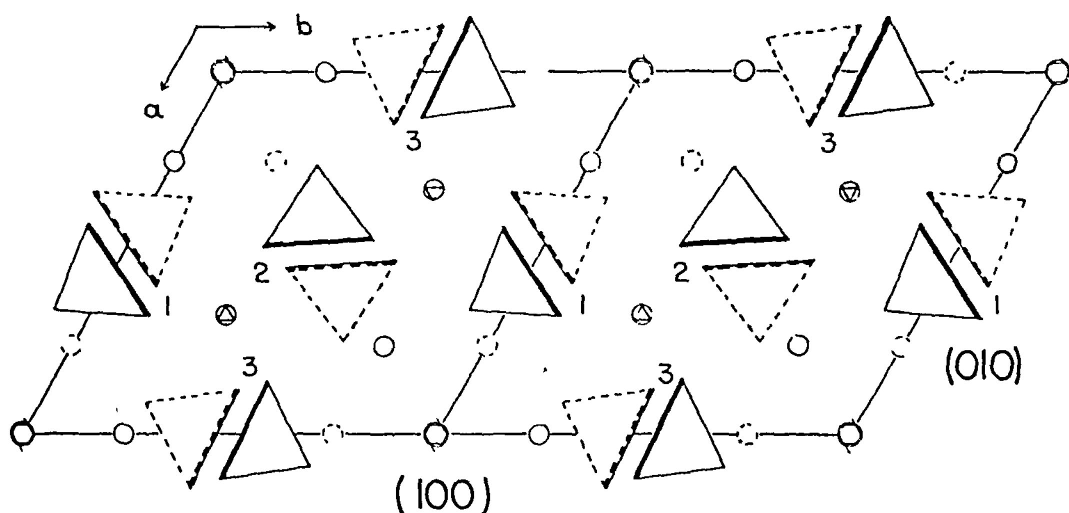


FIGURE 6 Basal projection of the apatite structure. The triangles are the projections of the PO_4 tetrahedra Their horizontal edges are marked by the heavy lines. The dashed PO_4 and Ca ions lie $c/2$ higher than those with the solid outline.

A basal projection of the apatite structure is shown in FIGURE 6. The PO_4 tetrahedra are arranged in pairs with their horizontal edges (heavy outline) on the horizontal symmetry planes. The horizontal edges of the tetrahedra 2 are not parallel to the face (100), but are rotated a few degrees. There is no vertical symmetry plane perpendicular to the prism surface, which is in accordance with the conditions of the apatite space group $P\ 6_3/m$.

It is quite plausible that the surface forces have some differentiating influence on the adsorption upon the prism face (100) of CO_3 groups with orientation 2, as compared to CO_3 groups with orientations 1 and 3. The difference may consist in the numbers of included groups (for example, positions 2 are less frequently occupied by CO_3 groups), or the difference may be in the tilt angle between the groups and the horizontal (for example, groups 2 being steeper than groups 1 and 3). Omitting some CO_3 groups in position 2 or increasing their tilt angle would lower the refractive index for vibrations per-

Where do we suggest placing the carbonate groups in the apatite structure? The oxygen tetrahedra of the PO_4 groups are nearly regular tetrahedra. Two of their faces are vertical, meeting in a vertical edge, and the other 2 faces are inclined, meeting in a horizontal edge. The tilt angle of the latter 2 faces is about 35° from the horizontal upward and downward. The CO_3 groups can fit easily into the spaces of the PO_4 groups, which they displace. The edge length (O to O distance) of the CO_3 triangles is about 2.15 Å, while the triangular faces of the PO_4 groups have an edge length (O to O distance) of about 2 50 Å. The tilt angle for equally tilted CO_3 groups, as calculated above, is not far from the tilt angle of the 2 inclined faces of the PO_4 groups. The CO_3 groups randomly will take the upper and the lower positions, as there is an equal chance for both orientations, in accord with the horizontal symmetry planes of the apatite structure.

The fact that the carbonate groups in the apatite are oriented is no reason not to consider them an impurity. There are many examples of large dye molecules being orientedly included in simple inorganic salts.[11] Furthermore, there is no need of picturing the CO_3 groups as being adsorbed lying flat on the prism faces of the growing apatite crystal These groups, instead, attach themselves with 1 corner or 1 edge to the surface while the other end sticks out toward the solution. In the growing calcite crystal the carbonate groups are also not positioned parallel to the growing rhombohedral face, but inclined at about 45° to it; and the basal plane, to which all CO_3 groups are parallel, is a face that is developed only rarely. If all CO_3 groups in the above-mentioned dahllite were adsorbed and included parallel to the prismatic planes, we should expect them to contribute a birefringence of about +0.019 instead of the observed −0 012.

In my opinion, the situation will be represented best by assuming that the carbonate in the apatite is present as an impurity that is caught in the growing apatite on account of its affinity to one of the ions in the structure (Ca) and not because it enhances the stability of the apatite structure. It is even caught when, in higher concentration, it destroys the apatite structure and forms an amorphous aggregate with the phosphate.

Optical Anomaly in the Carbonate Apatites

I am in possession* of some francolite crystals from the Harvard Museum (No H *89 618*) They are hexagonal prisms, about 1 mm. across and 1.5 mm. long; the prism faces are striated parallel to *c*, and the termination is an irregular base without pyramid. A thin section, about 100 μ thick, cut perpendicular to *c* through the middle of the crystal, when examined in polarized light, shows a uniaxial center surrounded by 6 sectors that are anomalously biaxial Their birefringence in this basal section is very low. The extinction, and with it, the axial plane in a sector is not parallel or perpendicular to the prism face. In the adjacent sectors the extinction is rotated 60° so that in the cross section the axial planes of the 6 sectors are oriented in a pinwheel fashion in accordance with the hexagonal *c*-axis 6_3 of the apatite. When the microscope stage is rotated, 2 opposite sectors always change color simul-

* Through the kindness of Clifford Frondel, Harvard University, Cambridge, Mass.

METABOLIC DISTURBANCES IN TOOTH FORMATION

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In noting the passage of more than one hundred years since the first detailed clinical description of developmental tooth defects was offered by Hutchinson, we believe that perhaps the most gratifying single advance has been the shift of emphasis from mere academic curiosity about this category of deformity to serious concern. Thus, where major attention had been devoted previously to advancing and popularizing different theories of etiology such as Magitot's eclampsia,[1] Harlan's eruptive diseases of childhood,[2] and Hutchinson's congenital syphilis,[3] there is today a realization that deficiencies in growth and development, wherever they may occur, can no longer be viewed as isolated phenomena. Furthermore, it is apparent that the seemingly endless array of chemical, physical, and biological factors shown to be related causally to enamel and dentin defects, rather than complicating our understanding of etiology, make quite prophetic the words of G. V. Black that "there is no special form of disease that is especially blamable for this affliction, but that any form of disease that seriously interferes with nutrition is liable to bring about this result."[4] Today this concept may be modified further to suggest that abnormal tooth formation is a generally nonspecific phenomenon and can be related to a variety of local and systemic disturbances, any of which, depending upon their severity and the degree of tissue response, may result in defective enamel and dentin, either of the so-called hypoplastic or hypocalcified variety. However, despite our increasing knowledge of etiological factors, the mechanism of development of dental defects continues to be understood poorly. This is not surprising, since most of the earlier studies seeking to define pathogenesis were based upon examination of ground sections of abnormal human teeth and, consequently, resulted in the advancement of theories that were as confusing as those that were advanced to clarify the subject of etiology. For example, Zsigmondy[5] attributed enamel hypoplasia to a decay of ameloblasts; Berten[6] supported a concept of poor calcification along the striae of Retzius; Walkhoff[7] believed in ameloblastic injury by microorganisms; and Gottlieb[8] emphasized ameloblastic degeneration and crumbling of "elementary substance" due to defective deposition of calcium salts. It bears reminding, however, that these views did not have the benefit of our present-day concept of the cellular or organic matrix phase of tooth formation as differentiated from the maturation or calcification process.[9] Theories notwithstanding, there remains the important question whether it may be assumed that the effects of many known etiological factors and agents are mediated through common pathways of influence or metabolic dysfunction or whether we must regard these as distinctly different processes. Whereas gross anatomic appearances of enamel hypoplasia might favor the latter view, observations at the microscopic level well justify the assumptions, for example, that chronic fluorosis induces its peculiar type of enamel lesion in much the same manner as rickets produces its associated dental deformity, or that syphilis induces its peculiar

pendicular to the edge In general, when such anisometric anisotropic groups as the CO_3 groups are adsorbed and included in a growing crystal, we expect, whatever the specific mechanism is, the faces of the growing crystal to modify within their sectors the hexagonal symmetry of the crystal, superimposing upon it its lower two-dimensional symmetry. If the apatite space group had a vertical symmetry plane perpendicular to the surface, then we should expect the extinction and the axial plane to be parallel or perpendicular to the surface.

On account of the horizontal symmetry plane there is an even chance for the CO_3 groups to occupy the upward or the downward tilted position. Accordingly, sections cut parallel to the *c*-axis exhibit in polarized light extinction parallel and perpendicular to *c* The central core of the francolite crystal remains uniaxial. Growth of the crystal in direction of the *c*-axis by apposition on the (001) face can retain the hexagonal symmetry, as the 3 positions have equal inclination to the surface and the same chance of being occupied by a CO_3 group

Acknowledgment

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METABOLIC DISTURBANCES IN TOOTH FORMATION

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In noting the passage of more than one hundred years since the first detailed clinical description of developmental tooth defects was offered by Hutchinson, we believe that perhaps the most gratifying single advance has been the shift of emphasis from mere academic curiosity about this category of deformity to serious concern. Thus, where major attention had been devoted previously to advancing and popularizing different theories of etiology such as Magitot's eclampsia,[1] Harlan's eruptive diseases of childhood,[2] and Hutchinson's congenital syphilis,[3] there is today a realization that deficiencies in growth and development, wherever they may occur, can no longer be viewed as isolated phenomena. Furthermore, it is apparent that the seemingly endless array of chemical, physical, and biological factors shown to be related causally to enamel and dentin defects, rather than complicating our understanding of etiology, make quite prophetic the words of G. V. Black that "there is no special form of disease that is especially blamable for this affliction, but that any form of disease that seriously interferes with nutrition is liable to bring about this result."[4] Today this concept may be modified further to suggest that abnormal tooth formation is a generally nonspecific phenomenon and can be related to a variety of local and systemic disturbances, any of which, depending upon their severity and the degree of tissue response, may result in defective enamel and dentin, either of the so-called hypoplastic or hypocalcified variety. However, despite our increasing knowledge of etiological factors, the mechanism of development of dental defects continues to be understood poorly. This is not surprising, since most of the earlier studies seeking to define pathogenesis were based upon examination of ground sections of abnormal human teeth and, consequently, resulted in the advancement of theories that were as confusing as those that were advanced to clarify the subject of etiology. For example, Zsigmondy[5] attributed enamel hypoplasia to a decay of ameloblasts; Berten[6] supported a concept of poor calcification along the striae of Retzius; Walkhoff[7] believed in ameloblastic injury by microorganisms, and Gottlieb[8] emphasized ameloblastic degeneration and crumbling of "elementary substance" due to defective deposition of calcium salts. It bears reminding, however, that these views did not have the benefit of our present-day concept of the cellular or organic matrix phase of tooth formation as differentiated from the maturation or calcification process.[9] Theories notwithstanding, there remains the important question whether it may be assumed that the effects of many known etiological factors and agents are mediated through common pathways of influence or metabolic dysfunction or whether we must regard these as distinctly different processes. Whereas gross anatomic appearances of enamel hypoplasia might favor the latter view, observations at the microscopic level well justify the assumptions, for example, that chronic fluorosis induces its peculiar type of enamel lesion in much the same manner as rickets produces its associated dental deformity, or that syphilis induces its peculiar

"screwdriver incisor" and "mulberry molar." Studies of both human and experimental material, representing a wide range of systemic diseases and conditions,[10-14] have shown that the earliest histopathological changes in enamel

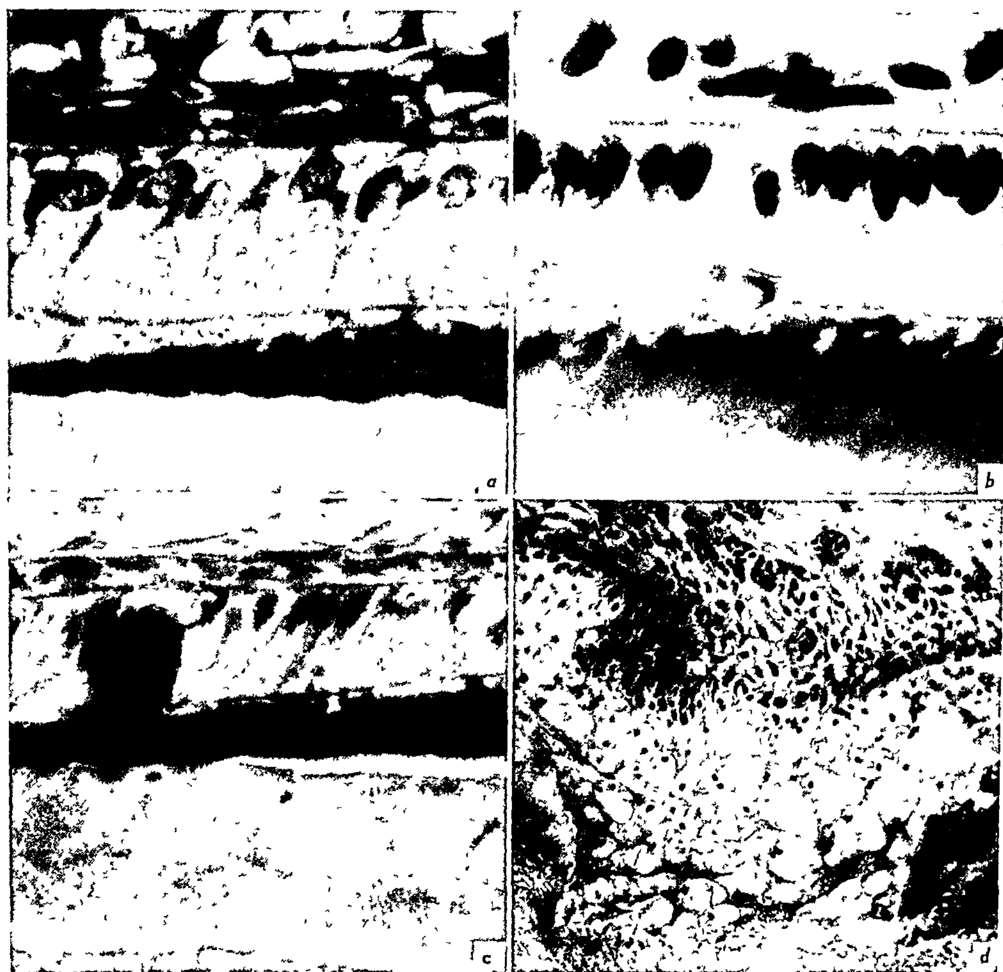


FIGURE 1 (*a*) Early cystic changes in ameloblasts of deciduous cuspid from a breech-delivered 3½-day-old baby Multiple congenital defects were found at autopsy. Maternal history of intermittent vaginal bleeding during second and third trimesters

(*b*) Vacuolization and nuclear pyknosis in ameloblastic zone of deciduous incisor from a 2-day-old cesarean-delivered baby Maternal history of toxemia of pregnancy

(*c*) Exudation of enamel-like material from vacuolated ameloblasts of a deciduous canine from a 6-day-old term-delivered baby Occult spina bifida found at autopsy. Maternal history negative

(*d*) Zone of arrested amelogenesis in mandibular deciduous first molar showing edematous change in area of defect. Multiple congenital abnormalities were found at autopsy. Maternal history of hypochromic normocytic anemia

hypoplasia are characterized by the appearance of globular, enamel-like material subjacent to and within the cytoplasm of ameloblasts. As this lesion progresses, there is beginning vacuolization of the cells and, finally, complete cystic degeneration with the onset of edematous change It is significant that the typical sequence of histopathological events, illustrated in FIGURE 1, is

equally applicable to all the cited experimental procedures, whether they be tuberculosis in mice and guinea pigs, rickets in dogs and swine, or parathyroid deficiency and fluorosis in rats. Nevertheless, it should be kept in mind that, although different disease processes may mediate their effects on the teeth through common pathways of metabolic dysfunction, it is possible that the high degree of differentiation of ameloblasts and odontoblasts predisposes them to a ready and similar response to a variety of injurious agents.

The established fact that alterations in metabolic processes, regardless of how initiated, can affect tooth development adversely, may have significant implications in terms of the relationship of microscopically occurring tooth defects to caries susceptibility.[15,16] Certainly, the peculiar patterns of anatomic bilateral distribution of carious lesions in man favor such a hypothesis.[17,18] Another important consideration of developmental dental defects is their utilization as a valuable tool for the pictorial recording and timing of systemic disturbances in man. In this regard, it should be emphasized that any observed disturbance in the highly specialized function of odontogenesis might be indicative of an unfavorable response of nondental tissues undergoing simultaneous development. Although defects in tooth development usually are thought of as postnatal in occurrence, there is an increasing awareness of their particular pertinence to the field of congenital anomalies, where prenatally occurring dental defects may not be only indicative of a widespread fetal response to adverse maternal influences, but also provide a method for evaluating the nature and mode of maternal-fetal disturbances.

In the course of a series of studies, which permitted the tracing of the pathogenesis of enamel hypoplasia from its earliest manifestations to the end result of clinically demonstrable defects, a number of observations of particular relevance to the metabolic nature of this abnormality were made.[19,20] As indicated previously, since many types of prenatally and postnatally occurring systemic disturbances may elicit a similar ameloblastic response, it is conceivable that certain common denominators of disease, such as fever, anoxia, and other metabolic influences may be the actual exciting agents or trigger mechanisms. Such a relationship was suggested in the case of fever[21] and alloxan diabetes[22] (FIGURE 2). In the first study on the effect of pyrexia on the course of pregnancy, the rats used were followed at various stages of gestation in order to determine the influences that this might have on the developing teeth of both maternal and fetal organisms. The duration of fever (induced by means of an incubator equipped with ventilation and humidity controls) ranged from 3 to 48 hours. Striking changes, not correlated with the stage of pregnancy when the fever occurred, were noted in the mothers. These ranged from early ameloblastic injury to complete cellular degeneration and arrest of enamel matrix formation. Identical enamel abnormalities, plus dentinal disturbances, were observed in the offspring of the pyrexic rats. In the case of diabetes, an experimental design was followed in which pregnant rats were injected intraperitoneally with 155 mg. of alloxan per kilogram of body weight.

Dental findings in both mothers and offspring were similar to those observed in the pyrexia study and showed no correlation with either the gestational period of drug administration or the severity of hyperglycemia. Since temperature recordings were not made of the *in utero* young in the first study, and

since the fetuses of the alloxan diabetic rats showed no clinical, laboratory, or histological evidence of pancreatic dysfunction, the specific nature of the metabolic disturbances causing the dental defects in both studies could not be identified.

Although the foregoing studies soon led to a consideration of the role of the placenta as a barrier to or pathway for specific bacterial and viral agents, the results of these later investigations could not rule out the fact that dental and other congenital defects still might be caused by some metabolic dysfunction incident to the maternal infectious disease rather than to transplacental passage of microorganisms per se. Whereas congenital syphilis has received a major share of attention in the earlier literature,[23,24] recent years have seen an

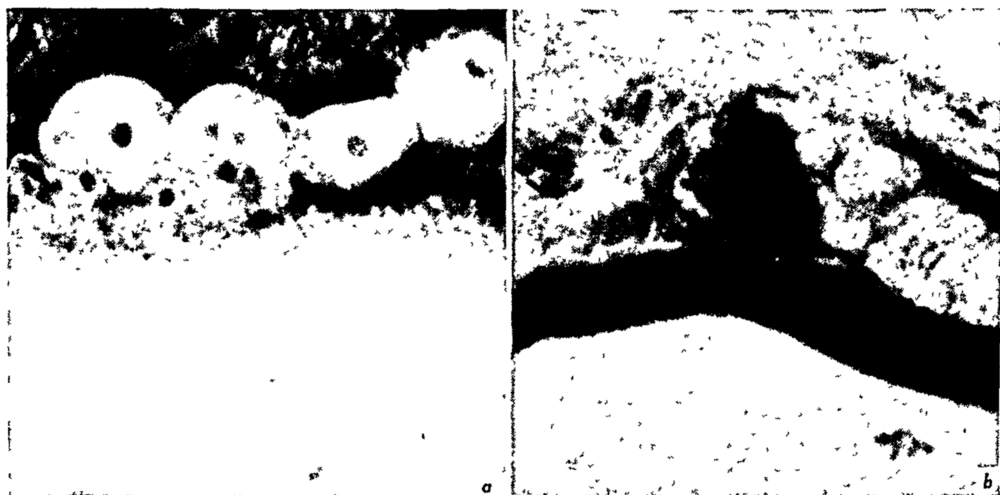


FIGURE 2 (*a*) Mandibular left incisor of pyrexic rat showing multiple, cystlike formations in ameloblastic zone. Similar changes were noted in the developing incisors and molars of offspring.
(*b*) Exudation of enamel-like material from cystic ameloblastic zone of a mandibular molar taken from a 1-day-old young of an alloxan-diabetic mother Similar changes were noted in the incisors of parent animals

increasing awareness of the role of viruses in the pathogenesis of congenital defects. While rubella, mumps, varicella, herpes zoster, and rubeola all have been implicated by clinical observation,[25] definite evidence of intrauterine infection by the viral agents of these diseases still is lacking.

In the course of experiments with vaccinia virus, striking abnormalities were noted in the developing teeth of female rabbits that had been inoculated intravenously with 1-ml. doses of an egg-adapted dermal lymph strain of virus in suspension, titering 10^{-4} to 10^{-6} (FIGURE 3).[26] Despite inoculation during varying stages of pregnancy and the demonstration of a rapid onset of generalized infection, no corresponding evidence of viremia or dental abnormalities could be found in the cesarean-delivered offspring. In speculating on the pathogenesis of the maternal tooth defects, it was of interest to note, not only their peculiar pattern of formation, but also the absence of associated Guarnieri inclusion bodies. Since the dental deformities resembled, in almost every morphologic detail, those described in parathyroidectomized,[27] hypophysec-

tomized,[28,29] and magnesium-deficient rats,[30] there was some reason to conceive of a common denominator that might explain pathogenesis in all cases. Most probable, on the basis of the buckled, wavelike appearance of the abnormalities, was some interference with the eruptive process. This impression, which paralleled the suggestion of Schour and Van Dyke[29] that continued dentin formation at the basal ends of the incisors, coincident with retarded eruption, was the responsible factor, was confirmed by the finding of a marked retardation in the eruption rate of the incisor teeth of vaccinia-inoculated rabbits as

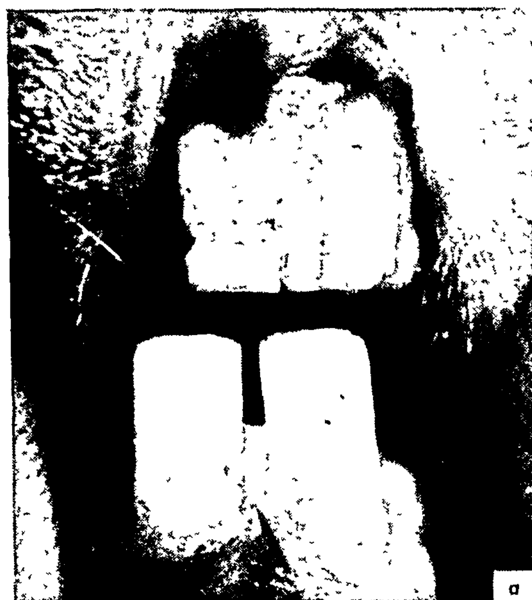




FIGURE 3 (*a*) Aplastic incisor of rabbit infected with vaccinia virus
(*b*) Buckled, wavelike appearance of incisor of rabbit inoculated with vaccinia virus 196 days before sacrifice
(*c*) Enamel aplasia and abnormal dentin formation in basal region of maxillary second premolar of rabbit inoculated with vaccinia virus 100 days prior to sacrifice A prominent incremental line separates dentin into 2 layers differentiated chiefly by change in tubular direction Osteoidlike dentin is noted to the right of pale-staining zone of enamel aplasia

compared with control animals. Assuming that the dental abnormalities in vaccinia-inoculated rabbits were related to some metabolic dysfunction incident to the disease rather than to the infection per se, the question still remains as to why fetal tooth development also was not affected. Thus, we continue to seek information on the pathogenesis of congenital abnormalities in the presence, as well as absence, of viral and bacterial diseases.

Further emphasizing the role of metabolic disturbances as a by-product of maternal infection, Gillman and his co-workers[31] advanced the hypothesis that many congenital defect syndromes are causally related to the combination of plasma proteins with abnormal metabolites derived from tissue breakdown in disease. Thus, various disturbances, including malnutrition and certain virus diseases, conceivably could injure tissues by depriving them of certain essential nutrients, as well as flood the circulation with particles derived from the abnormal metabolism. Such particles then could be absorbed by the albumin fraction of plasma protein and interfere with metabolic processes. Inasmuch as it had previously been demonstrated that trypan blue was absorbed by plasma albumin, several investigators studied and reported on the development of congenital malformations in the offspring of rats and mice inoculated with this dye.[31-33] Although various abnormalities were described, including spina bifida, rudimentary tail, the absence of eyes, and hydrocephalus, the developing teeth generally were spared. While it was somewhat surprising at first to find that this example of an experimentally created metabolic disturbance did not include defects of tooth development among the varied assortment of other anomalies, it should be emphasized that, just as different environmental factors may initiate identical congenital abnormalities, so may a given agent cause different types of developmental arrest.

Summary

From the foregoing it is evident that ample clinical and experimental evidence exists to suggest that developmental tooth defects are generally nonspecific in nature and can be related to a wide range of systemic disturbances, any of which, depending upon their severity and the degree of tissue response, might result in defective enamel and dentin. In addition, microscopic studies have made possible the tracing of the pathogenesis of enamel hypoplasia from its earliest cellular manifestation to the end result of clinically demonstrable defects.

Despite the advances in the understanding of pathogenesis and etiology of abnormal tooth development, they relate primarily to the postnatal period, and only limited information is available on the subject of prenatal influences. In order to add to the meager fund of knowledge on the relationship of various metabolic and viral agents to congenital abnormalities of the teeth, a series of studies was undertaken. These studies provided evidence to suggest that similar mechanisms of pathogenesis and etiology exist as they do in the case of postnatally occurring disturbances.

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DENTAL CARIES AND HOST METABOLISM*

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Until recently, most dental educators taught that the tooth is metabolically inert. Because of the absence of a vascular circulation, it was held that the hard tissues of the tooth were beyond the pale of metabolic exchange. Dental texts generally have given little consideration to the likelihood that intrinsic forces may affect the susceptibility of the tooth to decay. Certain investigators, notably Bodecker,[1] Beust,[2] and Fish[3] had presented their evidence and views that the posteruptive changes in the tooth may serve to retard decay, but their interpretations were not widely accepted at the time of their pronouncements. With the development of newer techniques of investigation, it has been possible to demonstrate changes that previously could only be postulated and to substantiate the basic principle that the tooth is metabolically reactive.

Guttorm Toverud, in the National Research Council's "Survey of the Literature of Dental Caries,"[4] makes the statement: "There can be no doubt that vital reactions take place in the dentin of erupted teeth." Continuing, he states, "The enamel of the erupted tooth with living pulp may be considered as a living structure capable of undergoing certain physiologic changes from within and from without. The changes so far demonstrated, however, are not of the nature of metabolic processes in the ordinary sense."

Whatever is believed to comprise "metabolic change," it has been shown that ion exchange occurs continuously within the enamel and the dentin of sound teeth,[5] and that intact enamel is readily and rapidly permeable to ions and even to large molecules.[6] Physiological mechanisms serve to lead the tooth substance continuously toward a state of adjustment and readjustment to substances dissolved in the body's extracellular fluids.‡ Such changes are associated with histological, physical, and chemical changes within the tooth, and with altered susceptibility to dental caries.

In this report it is planned to re-state and clarify the theorem, already outlined many years ago by Beust,[2] relating to the manner in which a tooth may protect itself against decay through physiological agencies. The substance of the theorem has been drawn from the publications of other students of dental physiology and from my own observations, both published and unpublished, during the past thirty-three years. No consideration will be given to the role or the manner of action of caries-promoting agencies working within the oral cavity. Although relevant, such consideration goes beyond the scope of this discussion.

The concept to be presented rests on the premises that the tooth at the time of eruption is an incomplete structure; that it undergoes progressive changes

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‡ Some of the evidence for posteruptive chemical and physicochemical changes in the erupted tooth have been reviewed in a previous publication.[7]

so long as it remains vital; and that if the tooth can resist carious destruction during its first few posteruptive years, it may tend to reach a state of essential immunity to decay. Although the posteruptive changes of maturation in the tooth are manifested in each of its major components, consideration here will be directed primarily to those seen in the dentin.

It will be recalled that the odontoblasts that line the pulp chamber of the vital tooth remain physiologically active throughout the lifetime of the individual. Arising initially at the dentinoenamel junction of the tooth bud, as a component of its growth they migrate away from that junction mesially with progressive encroachment on the pulp chamber while retaining their connection with the junction through the dentinal fibril or tubule. Prior to eruption, the size and contour of the crown of the tooth have been fixed by the enamel that already has been deposited. The bulk of the dentin, on the other hand, increases progressively posteruptively, at the expense of the pulp chamber.

The cells that comprise the odontoblastic layer are responsive to stimuli from the exterior of the tooth. Such stimuli are conveyed from the surface of the enamel to the odontoblast through its dentinal tubule. The response of the odontoblast to such stimuli determines the posteruptive changes in the dentin. Such stimuli may be either physiological or pathological. Among physiological stimuli would be included pressure from occlusion or attrition, thermal changes, physicochemical forces induced from osmotically hypertonic foods, and chemical effects from ingested substances that might react with the tooth's constituents.

The odontoblast may respond to external stimuli in various ways. The normal response to shock stimulus is the deposition of inorganic material in the transmitting tubule, appearing first at a point about midway between the terminus of the viable portion of the damaged tubule and the pulp boundary. This will lead toward the sclerosis and subsequent lessened permeability of that tubule. If the stimulus does not exceed the capacity of the odontoblast for physiological response, it also will lead toward more rapid growth of the odontoblast toward the pulp, with consequent thickening of the layer of secondary dentin and with increased insulation of the pulp in corresponding measure. If the stimulus exceeds the capacity of the odontoblast for normal response, it may lead either to the formation of irregular secondary dentin or to death of the odontoblast. Such an odontoblastic response has been observed to develop rapidly with depletion of the tissue reserve of vitamin C, with recovery if the deficiency is corrected in time.[8]

The pattern of sclerosis within tracts of adjacent dentinal tubules may be demonstrated in the polished tooth half through its imbibition of stain or of radioisotope. The greater the sclerosis, the less the porosity of the region. The dentin of the newly erupted tooth will imbibe stain freely and rather uniformly throughout its substance, with accentuation adjacent to the pulp chamber and at the dentinoenamel junction. In contrast, the sound tooth that has resided in the mouth for several years will show diminution or absence of staining of certain tracts of tubules as contrasted with the total dentinal mass. The sound tooth from the mouth of a mature individual will tend to reject the stain throughout, as a measure of the cumulative sclerosis of its tubules.

Dentinal sclerosis tends to appear first in regions where the enamel is thinnest: for example, beneath developmental pits and fissures When such sclerosis does not develop promptly, caries is prone to gain a foothold in these same sites. With adequate sclerosis of the tubules underlying an actual or a potential enamel defect, such tubules will be sealed off completely, and the dentinal hardness may come to approximate that of sound enamel. Even though definite caries of the enamel may be present, the initiation of adequate sclerosis of the underlying dentin may preserve it from attack. In a tooth where dentinal caries already has made notable inroads, a change in its sclerosing propensities can bring about hardening of the exposed dentin, with complete arrest of the caries process and with freedom from later caries advance, even though no local operative or other dental therapy has been used. If such a tooth later becomes carious, the caries will develop in another area rather than in any region that has undergone sclerosis. When the sclerotic process is only partially effective and caries continues to spread pulpward, sclerosis of the surrounding dentinal tubules may prevent its spreading laterally. At the underlying pulp margin, the odontoblasts may lay down significant amounts of irregular secondary dentin that will serve in some measure as a barrier against caries invasion of the pulp.

The fact that effective dentinal sclerosis will develop in one person's teeth whereas in another's mouth it is insufficient to prevent caries must be explained on the basis of differences in body efficiency. For the body as a whole and for its constituent tissues, the zone between passable and optimal levels of functioning is broad. This is true not only from one person to another, but in the same individual from one period to life to another. If conditions of living are improved, levels of physiological performance may be raised in many regards.[9] The levels of various electrolytes in the body are in a state of flux[10] The homeostatic mechanisms can adapt themselves to the maintenance of life either in ideal conditions or under adversity, but not without subtle changes in the various body functions. To what extent the protective effect of improved diets on the preservation of the teeth from decay relates to the responsiveness of the odontoblast itself and in what measure it may reflect minor shifts in the electrolyte exchange between the body fluids and the tissues remains a problem for further study.

My own interest in the problem of dental caries was aroused by the late Charles L. Drain, the pedodontist who provided dental service among the child patients at the Children's Hospital at the State University of Iowa, Iowa City, Iowa. Drain had noted that some patients with open, untreated cavities would develop arrest of caries within a few weeks of the beginning of their period of hospitalization. He defined arrest of caries as the change of consistency of the exposed dentin from easy penetrability by the exploring tine to stony hardness.[11] His clinical interpretation was in accord with the histologic findings reported at about that time by Beust,[2] and is compatible with the condition described by Bodecker[1] as "protective metamorphosis," and by Fish[3] as "calcified tissue of repair." Microscopic examination of extracted teeth that had developed arrest of decay showed that the hardness was associated with sclerosis of the exposed dentinal tubules

The study of these children with arrested caries led to prolonged surveys of

the serial status of the teeth of many hospitalized children.[12] The findings were contrasted with those from similar surveys of dental records of children who came recurrently to the university's dental infirmary for the school-sponsored examination of their teeth,[13] and also among children living in state-supported custodial institutions.[14] Among the hospital child population, it was found not only that open cavities became sclerosed and caries inactive, but also that the incidence of new caries lesions was much reduced among those children who remained under the regimen of hospital life, as compared with such incidence among the control groups. The hospital diets for all types of patients were similar in that each of them included all recognized nutritional essentials in liberal amounts, and in that their daily ingestion was supervised by well-qualified dietitians The more rigid the day-by-day supervision of the intake of the prescribed foods, the greater the arrest of caries and the less the development of new dental lesions. For this reason, rather than because of the avoidance of sugar, the lessening of caries was most notable among the children with diabetes mellitus.[15] However, similar response of noteworthy degree was observed also among other groups of children whose diets were nutritionally complete, but whose use of sugar was not proscribed.[16]

The Iowa clinical studies of caries control carry weight particularly because of the following factors characterizing the routine of dental examination, maintenance of records, and program of therapy:

(1) There was opportunity for recurrent examination of many individual children for periods of months and years.

(2) The dental records defined accurately the serial changes for each tooth surface and for each identifiable dental abnormality.

(3) The method of recording the dental findings permitted comparison as to the occurrence, timing, nature, position, size, and physical consistency of the individual lesions of the enamel and of the dentin from one examination to the next.

(4) Because of the frequency of dental observation it was safe to leave cavities unfilled and to omit local treatment, except under circumstances where such delay would endanger the tooth or interfere with mastication. Such being the case, it was the custom to delay operative or other local dental therapy until the course of the caries had been determined through serial observations of the lesions. So-called prophylactic fillings seldom were used.

Due to the conditions described, it was possible to follow each new caries lesion from its incipiency throughout its progress or its arrested state for periods of years, and to draw an accurate profile as to the rate of caries advance for each lesion, its degree of regularity of progression, and the cumulative caries score for individual mouths. Only through studies permitting such exactitude of record could the true course of caries have been determined. No direct comparison can be made between massed dental data relating to rates of caries progression and those derived from such individual serial observations as were made in the Iowa studies.[17-19]

On the basis of the Iowa clinical studies, it was concluded that dental caries in children's teeth could be avoided or arrested through the regular ingestion of diets of high nutritional worth. Similar conclusions were reached by Howe and his colleagues at the Forsythe Clinic.[20,21] Shaw cites still other correspond-

ing diet studies.[22] The work of East[23] and of Mellanby[24] emphasized the role of vitamin D in the protection of the teeth. Howe's attention had been directed more toward the role of vitamin C.[8] Evidence supported the view that each of these agents played an important part in the maintenance of normal tooth physiology. The Iowa studies were not concerned with the role of any particular factor. The view was held that the objective should be the promotion of optimum physiological functioning of the organism in all regards, without overemphasis on any organ, tissue, or function to the possible disadvantage of another. The diets prescribed for the children in the Iowa studies were higher in many nutritional essentials than were those commonly eaten by the average child population.[16] This was true particularly of the level of ingestion of protein, of calcium, and of the various vitamins While refined foods were not proscribed, native foodstuffs were favored as sources of the essential nutrients. Levels of intake were in accord with the recommendations of the National Research Council, without excessive intake of any nutrient.

Possibly specific studies might serve to delimit the factors needed by the tooth. No doubt there are agencies other than those relating to the ingestion of foods which affect the over-all efficiency of homeostasis Surely chronic emotional tensions cannot be overlooked in such a review. The principal objective, however, should be the provision of optimal facilities for the promotion of over-all health of the child, regardless of his propensities for dental caries. With such provision, physiological mechanisms will be bolstered, and good should accrue to the child in many respects. Lessening of caries should be only one component of such benefit.

The illustrations that follow are offered as evidence of posteruptive changes in the dentin. Prints of photographs of halved, polished extracted teeth are shown together with the radiosulfur autoradiogram of the same. The procedure employed for autoradiography has been described elsewhere.[25] The polished longitudinal half-section of a tooth is immersed in a solution of suitable radioisotope until the tooth substance has become radioactive, then the surface is exposed to photographic film in darkness until the film is exposed sufficiently. After photographic development, the film then is used as a source for photographic enlargement. Sulfur-35 has been the radioisotope used in the studies to be reported, because it is a weak beta-emitter, and the autoradiographic image represents only surface characteristics of the specimen.[26] Those regions of the tooth that are most porous take up the most isotope. In the photographic print of the autoradiogram such areas appear white, while those with little uptake appear black. Cementum takes up radioisotope with great avidity, whereas enamel takes it up in minimal amounts. The uptake in dentin varies with the region of the tooth and with its degree of posteruptive mineralization. The lining of the pulp chamber and the odontoblastic layer usually appear white. The dentinal tubules tend to take up some radioisotope in the proximal third of their length, even in the adult tooth. The dentin of the young tooth tends to take up isotope in considerable amounts. The enamel of the young tooth likewise shows greater isotope uptake than does old enamel. Regions where tracts of dentinal tubules have undergone sclerotic change reject the isotope and appear similar in blackness to the enamel.

Each of the following figures shows corresponding enlarged prints of the photograph and autoradiogram of a given tooth.

FIGURE 1 shows a deciduous tooth, with occlusal and interproximal caries lesions, with penetration of the pulp chamber. Note the absence of any apparent sclerosis of the dentin. This exemplifies rampant caries, with absence of effective resistance to the spread of decay.

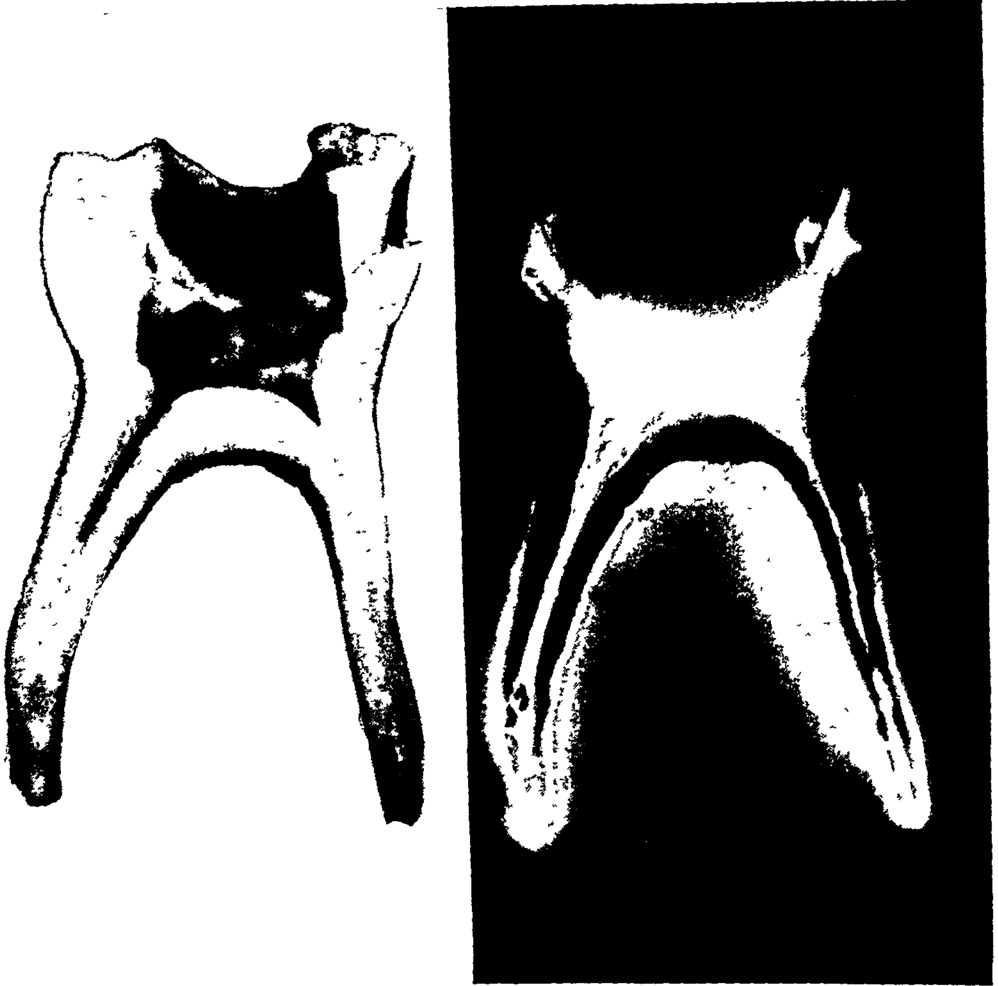


FIGURE 1.

FIGURE 2 shows another deciduous tooth, with caries lesions in two areas on the occlusal surface and a third at the contact point. Note the marked permeability of the carious dentin at the right. No sclerotic changes have developed under the region of interproximal caries. Beneath the occlusal caries at the left, however, there is evidence that the dentinal tubules have become impervious owing to secondary sclerosis of the tubules, with resultant partial arrest of the caries in that region.

FIGURE 3 illustrates caries beneath a leaky filling. Note the marked permeability of the dentin lying immediately beneath the filling, as evidence that active caries is present. However, the mesial two thirds of the underlying

tubules have rejected the radioisotope, offering evidence that the defense mechanisms of the tooth have been able to seal off more or less effectively the caries penetration from the pulp chamber. Likewise, considerable amounts of irregular secondary dentin have been deposited at the mesial surface of the sclerotic dentin. The thin line extending through the pulp horn to the dentinoenamel



FIGURE 2.

junction could well indicate an avenue of leakage through which the pulp has been infected.

FIGURE 4 depicts a large interproximal cavity in a tooth from a 65-year-old man. Note here that the dentinal tubules above and mesial to the cavity are virtually impermeable to the isotope. The caries probably is arrested, and may well have been at a standstill for many years. Note that the photograph, in contrast to the autoradiogram, offers poor evidence of the diminished permeability of the sclerosed dentin.

A tooth from the same mouth is shown in FIGURE 5. Note the interproximal and gingival caries lesions, with sclerosis mesial and lateral to the areas of dentinal caries. The lateral tracts have served to prevent lateral extension of the

caries at the dentinoenamel junction. Note the minimal degree of destruction of the dentin despite the penetrating lesion in the enamel. The incremental growth bands in the dentin show sharply in the autoradiographic print, even though they are not apparent in the photograph.

In FIGURE 6 is shown a gingival cavity with minor loss of dentin. A hairline tract of sclerosis extends to the pulp chamber, with notable amounts of





FIGURE 3.

irregular secondary dentin protruding into the latter. Note that the new dentin has not yet become well mineralized, as indicated by its heavy imbibition of radioisotope.

FIGURE 7 depicts a tooth from an elderly man. The enamel caries at each contact point has penetrated to the dentinoenamel junction and stained the underlying sclerosed dentin, but has failed to cause dentinal destruction. Note the greater degree of dentinal sclerosis in the broad tract of fibers extending from the region adjacent to the enamel defect at the left to the pulp chamber, with similar regions of sclerosis evident at the right under lesser breaks in the enamel. Small accretions of irregular secondary dentin can be seen at the termination of two of these sclerosed tracts.





FIGURE 4

FIGURE 8 shows interproximal and gingival caries in a tooth from an elderly man. Note the tracts of sclerotic dentin underlying the three superficially carious zones of dentin. Note also the sclerosis subjacent to the gingival region at the left, where retraction of the gingiva and erosion of the cementum had left the dentin exposed.

A noncarious tooth from the mouth of an elderly man is shown in FIGURE 9. The diffuse sclerosis of the crown and the irregular pattern of sclerosis of the root are evident both in the photographic and the autoradiographic print. Note that the penetrability of the dentin is greater adjacent to the pulp chamber than peripherally. Note also the degree in which secondary dentin formation has reduced the volume of the pulp chamber.





FIGURE 5.

The foregoing illustrations portray degrees of dentinal response to caries invasion ranging from absence of response to the complete arrest and prevention of caries. Teeth of elderly patients frequently exhibit evidence of arrested caries, often of many years' duration In young patients, such arrest is rare except in groups where betterment of living conditions has been imposed and maintained, as was true among our population of hospitalized children with prolonged hospital tenure

Orban, in the most recent edition of his text,[27] emphasizes that dentin is a vital tissue with capacity to react to physiological and pathological stimuli. He describes the manner in which damaged odontoblasts are stimulated to a

defense reaction in which hard tissue seals off the area of injury. He uses the term reparative dentin to describe this change. He also speaks of sclerotic dentin in the same relationship. It seems evident that his concept is not particularly different from that presented here, except that he refers to the dentinal changes as though they were pathological processes. To me it seems important that the process be recognized instead as a physiological defense mechanism,



FIGURE 6.

built into the odontoblast's manner of normal response and leading to a metabolically induced change in the tooth substance. The evidence presented here is designed to support such a concept.

In review, let us correlate the following three facts: (1) dental caries in the young person usually is progressive, leading toward the destruction of the tooth; (2) arrest of dental caries has been accomplished in the teeth of many children through measures directed primarily toward the betterment of the nutritional state; and (3) arrest of caries has been shown to be associated with sclerotic changes in the dentin. One is led to the conclusion that the posteruptive sclerotizing changes in the dentin represent a physiological defense against





FIGURE 7.





FIGURE 8

decay, and that such changes are the result of the role of metabolic mechanisms rather than by-products of pathological change. If such a viewpoint is correct, then it is important to emphasize the fact that the tooth possesses native defense mechanisms against the inroads of decay. Recognition of such truth should lead to positive methods of attack against dental caries as a phase of over-all health promotion. Attention then would be directed, not primarily

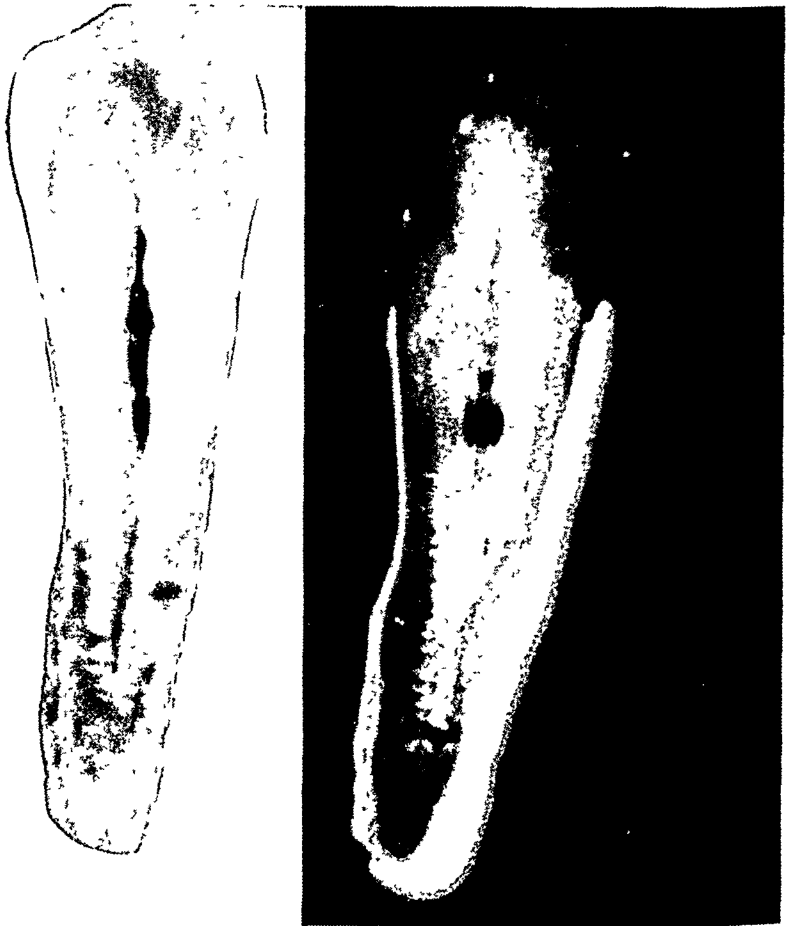


FIGURE 9.

toward the child's teeth, but instead toward the child himself and an analysis of his over-all propensities for health advancement.

Acknowledgement

I am indebted to the following personnel of the Veterans Administration Hospital, Coral Gables, Florida· Alvin Blum, Lee Starrett, Rudolph Sims, Jr., and Donald Osgood, who helped with the technical aspects of the autoradiographic work, and Al Weinberg, who prepared the photographic material and served as my advisor on photographic technique.

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Part III. The Salivary Glands

STUDIES OF SALIVARY GLAND PROTEASES*

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History

Proteases of salivary gland origin have been known since the time of Hufner in 1873. Later, Willstätter *et al.* (1929) described proteolytic enzymes in the salivary glands of the dog, pig, horse, and man. These investigators were concerned with placing the salivary glands in the general context of the digestive glands.

The direction of interest in salivary proteases changed with Lacassagne's (1940) discovery of morphologic sex differences in the submaxillary glands of mice. Lacassagne's findings focused interest on the hormonal control of salivary glands in rodents Hormonal control of salivary proteases in mice was first described by Junqueira *et al.* in 1949. Since then protein-splitting enzymes have been found in the submaxillary glands of other rodents.

Our studies were concerned with the characterization of the proteolytic activity of the submaxillary gland in the adult rat, with its developmental history and with its hormonal control. Parallel studies were conducted on the pancreas in order to compare the physiological role of the proteases of the two organs, and to compare a gland that exhibits sex differences with one that does not.

Characteristics of the Proteolytic Activity of Extracts of the Submaxillary Gland

The submaxillary gland of the albino rat contains considerable amounts of an enzyme system that can act on azocoll (azotized hide powder), casein, gelatin and benzoyl-arginine-amide (Sreebny *et al.*, 1955*a*). It does not act on chondroitin sulfate, gastric mucin, or native collagen. Similar extracts from rat spleen, liver, testis, kidney, muscle, and parotid and sublingual glands do not degrade azocoll. Pancreatic extracts activated by enterokinase, and extracts of the small intestine also degraded the dye-hide powder complex.

The enzyme activity of the submaxillary gland has it optimal *p*H at about 9.6. It is mildly sensitive to acids and markedly sensitive to alkali above its optimal *p*H. The optimum temperature is between 50 and 55° C. and enzyme activity is completely abolished by heating at 100° C. for 15 min.

Enzyme activity is increased by cysteine and ascorbic acid, and inhibited by iodine. Iodoacetic acid, $MgSO_4$, and trypsin inhibitor have no effect. Dialysis of the extract results in a decrease in enzyme activity due to the loss of some inorganic component.

The action of the enzyme follows the monomolecular reaction curve. For extracts prepared according to our assay procedure, the limiting substrate concentration is about 1.0 per cent casein. The breakdown of casein was measured as the liberation of tyrosine by the method of Kunitz (1947). The limiting

* This paper is based largely on studies carried out with the collaboration of Julia Meyer, J. P. Weinmann, and Erica Bachem

velocity was determined according to the method of Lineweaver and Burk (1934). The value for K_s was 0.086 per cent casein. With 1 per cent casein, decreasing extract concentrations liberated proportionately decreased amounts of tyrosine (Sreebny *et al.*, 1955*a*).

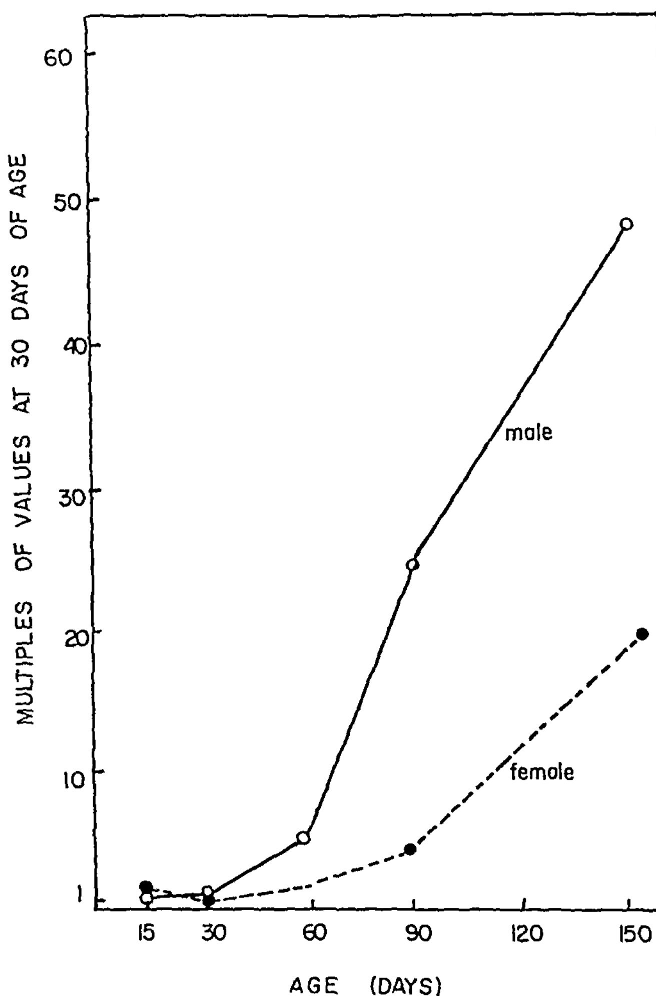


FIGURE 1 Protease activity of the submaxillary gland in rats of different ages.

Development of the Submaxillary Gland

Proteolytic activity of the submaxillary gland was assayed in male and female rats from 15 to 150 days of age using azocoll as a substrate (Sreebny *et al.*, 1955*b*; see also FIGURE 1).

In both male and female animals, the concentrations were stationary between 15 and 30 days, and there were progressively larger increases in the subsequent intervals. Concentrations in male animals increased forty-sevenfold over the 30-day values; those in female animals a little over twentyfold.

The degree of proteolytic activity in male animals was higher than that in females from 60 days on, the proportional difference was maximal at 90 days, when the proteolytic activity of male animals was about 5 times as high as that of the female ones. At 150 days, it was a little more than twice that of the females.

Puberty occurs between 60 to 70 days in these rats. These findings therefore suggested that the proteolytic activity of extracts of the submaxillary gland was under the control of sex hormones.

The submaxillary glands of most rodents, unlike the parotid and sublingual glands, show two prominent kinds of secretory cells, those of the acinar portion and those of the intralobular ducts. Stormont called them special serous cells (1932); the sexual dimorphism of the submaxillary glands in mice, discovered eight years later by Lacassagne (1940), concerns the intralobular duct portion of the gland. At both sites, increases in the number of secretory cells and in the number of granules in the cells occur between 15 and 150 days of age. But from 60 days on, the increase in cell number and granule content is far more conspicuous and rapid in the intralobular duct cells. Quantitative estimates in the rat show that the relative volume increase in intralobular secretory cells is from one tenth at 30 days of age to one fourth at 150 days of age for male animals, and of the same order for female animals. The acini and stroma decrease in proportion to the increase of the intralobular cells.

Since the proteolytic activity per unit duct volume increases with each successive age group, the present findings for the rat lead to the same tentative conclusion that Junqueira (1949) reached for mice, that the probable site of proteolytic activity is in the intralobular secretory cells.

Work on the effect of hormones to be discussed later points to the same conclusion. However, the question where the proteolytic activity resides is still undecided: for one reason, because the intralobular duct cells do not show the same high concentration of ribonucleic acid that we have come to associate with rapid protein synthesis.

The growth and size of the submaxillary gland slows between 60 and 90 days and resumes the prepubertal rates in the weeks following. The retarded growth during puberty is indeed typical for total body growth. The growth of the duct portion of the glands is retarded also, but much less than that of the entire gland. The proteolytic activity of the submaxillary gland, on the other hand, shows an accelerated increase between 60 and 90 days of age. The only examples of similar timing of growth are organs such as the prostate gland, that is, secondary sex organs that depend for their development on the male sex hormone.

It therefore appeared probable that the increase in the proteolytic activity of the submaxillary gland during puberty occurs under the influence of the male sex hormone.

In contrast to the findings for the submaxillary gland, the specific proteolytic activity of activated extracts of the pancreas remained unchanged with age (Sreebny *et al.*, 1958; see also TABLE 1) Its increase in size followed the same pattern as that of the submaxillary gland, so that the total proteolytic activity increased parallel with the size.

The pancreas in the adult rat weighs about 1 gm ; each submaxillary gland weighs about one fourth of this. The specific proteolytic activity of both submaxillary glands is about one tenth that of the pancreas. Thus the total protein splitting capacity of the former is about one fortieth of that of the pancreas. Unless it has some specific action, the contribution of the submaxillary gland

to digestion cannot be very great. Little is known, however, about the biological role of this enzyme system.

Effect of Hormones

Interest in the hormonal control of the submaxillary glands was inevitable once a morphologic sex difference was known.

Castration. There are conflicting reports in the literature concerning the effect of castration on the submaxillary gland. Raynaud (1944) showed that there was a significant loss in the absolute weight of the submaxillary gland and in the gland/body weight ratio in mice castrated at birth and sacrificed after 2 to 3 months. Sreebny *et al.* (1958), on rats castrated at 30 days of age, showed a significant loss in the absolute weight of the submaxillary gland, but not in the gland/body weight ratio.

TABLE 1
SPECIFIC PROTEOLYTIC ACTIVITY* OF THE SUBMAXILLARY GLAND AND PANCREAS IN NORMAL AND CASTRATE RATS OF DIFFERENT AGES

Age in days		Pancreas		Submaxillary gland	
		Normal	Castrate	Normal	Castrate
60	Average	13.0	10 23	0.13	0 11
	Range	12.3–13 9	6 2–12.5	0 07–0 22	0 09–0.14
	No. of animals	3	4	3	3
90	Average	13 0	13.37	0 45	0.16
	Range	11.4–15.0	12.3–14.6	0 23–0 52	0 09–0 26
	No. of animals	7	7	7	7
150	Average	11 8	10 99	0 56	0.30
	Range	10 7–12.9	9 6–12.2	0 36–0 65	0.22–0 40
	No. of animals	5	7	5	5

*O D mg N.

Baker and Pliske (1957) showed that castration of male rats resulted in an insignificant reduction in the absolute and relative weights of the pancreas, but had no effect on the parotid gland. This was also confirmed in our laboratory.

Proteolytic activity of the submaxillary gland was assayed in animals that were castrated at 30 days of age and allowed to live for between 30 to 120 days following the operation. Protease activity was not affected at 60 days of age. At 90, however, activity was cut down to one third of normal values and to one half at 150 days of age (TABLE 1).

These findings are in agreement with the earlier studies of Junqueira *et al.* (1949) on mice, which showed that the protease content of the submaxillary gland was reduced following castration.

In contrast to the findings on the submaxillary gland, castration had no effect on the proteolytic activity of the pancreas (TABLE 1; see also Sreebny *et al.*, 1958)

Replacement therapy. A significant role of the male sex hormone is seen also in replacement experiments.

The effect of hormone replacement on the proteolytic activity of the submaxillary gland, in so far as I have been able to determine, has not been tried on castrated animals. It has been done, however, on hypophysectomized animals.

Clark *et al.* (1957) found that in prepuberal, female, hypophysectomized rats the proteolytic activity and the morphology of the submaxillary glands could not be re-established with thyroxin or testosterone, or both.

However, in adult male rats we have found (Sreebny *et al.*, 1957) that the atrophy following hypophysectomy and the accompanying reduction in proteolytic activity of the submaxillary gland could in part be reversed following

TABLE 2

THE EFFECT OF HYPOPHYSECTOMY AND REPLACEMENT THERAPY ON THE PROTEASE ACTIVITY AND MORPHOLOGY OF THE SUBMAXILLARY GLAND

Treatment	Protease activity O D /mg N	Ratio of gland to body weight (× 1000)	Area occupied by intralobular ducts with secretory granules (in 1080 squares)
Normal	0 68 ± .066	0 192	275
Hypophysectomy	0 23 ± 047	0.111	None
Hypo + testosterone	0.36 ± .088	0 169	110
Hypo + thyroxine	0 37 ± 093	0 169	133
Hypo. + test + thyrox	0 62 ± 045	0 200	244

TABLE 3

THE EFFECT OF HYPOPHYSECTOMY ON THE SUBMAXILLARY GLAND OF THE ADULT MALE RAT

	Age (days)	Number in Group	Body Wt (Gm)	Gland dry Wt (mg)	Ratio ducts/acini	Protease activity
Normal	150	7	522	99	0 66	47 4
Hypophysectomized	150	6	371	51	0 32	5.66

the administration of testosterone and could be completely reversed with the additional administration of thyroxin (TABLE 2).

Hypophysectomy. Whereas castration led to a reduction to about one half in the proteolytic activity of the submaxillary gland, hypophysectomy led to more drastic reduction (TABLE 3).

Hypophysectomy led to (1) a decrease in the dry weight of the gland, (2) marked atrophic changes in the intralobular duct portion and a decrease in the intralobular duct granules, and (3) a decrease in the proteolytic activity of extracts of the submaxillary gland to about one ninth of the normal value (Sreebny, 1954).

These findings were later confirmed by Shafer and Muhler (1955) for the rat, and they agree with the morphologic findings described earlier in the mouse by Lacassagne and Chamorro (1940).

Barrett *et al.* (1955) and others have shown that the zymogenic cells of the pancreas underwent involution following hypophysectomy. Similarly, the

weight of the pancreas (Koster, 1930; Bryans *et al.*, 1952) and its proteolytic activity were markedly reduced (Baker *et al.*, 1956).

Baker and Pliske (1957) showed that implantation of pituitary glands restored the absolute and relative weights of the pancreas to nearly normal levels Excluding implantation of pituitary glands, attempts to restore the structure and function of the pancreas in hypophysectomized rats with replacement therapy were not completely successful.

Hypothyroidism. There is agreement among the various workers in the field that the submaxillary gland is under control of sex hormones. Whether the sex hormones play the major or a minor role, however, is a matter of disagreement.

Propylthiouracil (0.05 to 0.075 per cent) was placed in the drinking water given to adult male rats for varying periods of time.* Normal and pair-fed and pair-watered groups were used as controls.

After 2 weeks it was found that the absolute weight of the thyroid gland and the gland weight/body weight ratio in the animals given propylthiouracil was 3 times greater than their pair-fed or normal controls. The protein-bound iodine values of the serum decreased from a mean of 3.56 μg. per cent in the normal rats to 1.00 μg. per cent in the experimental animals. Thus there was a 70 per cent reduction in the amount of circulating thyroid hormone in the experimental animals Histological examination of the gland revealed acinar hyperplasia and a great diminution in the amount of colloid.

Propylthiouracil brought about a demonstrable but slight reduction in the absolute and relative weights of the submaxillary gland and the pancreas. These findings are in agreement with those of Grad and Leblond (1949) and Clark *et al.* (1957). Baker *et al.* (1957) came to similar conclusions for the parotid gland. The effect on the submaxillary gland therefore appears to be no different from that on the other exocrine glands

As regards the specific proteolytic activity we found, after two weeks, no change in either the pancreas or the submaxillary gland. This agrees with the data shown by Clark *et al.* (1957) in which a reduction of proteolytic activity does not occur before nine weeks. Townsend and Baker (1959) have shown that the amylolytic activity of the pancreas decreases six weeks following surgical thyroidectomy. Their results show that longer and complete deprivation reduces, not only the weight of the gland, but also its specific enzymatic activity. This was also evident from the need for thyroxin in addition to testosterone to restore full activity in the hypophysectomized animals. Our results demonstrate that, in a two-week period with a thyroxin level one third of normal, the proteolytic activity is unaffected. It remains to be seen whether the time factor or the dosage factor is responsible for the reduction observed by others and whether affects, when they appear, are of the same order in different exocrine glands.

We therefore feel that none of our own results nor the results in the literature justify the conclusion that the thyroid plays a specific role in the functioning of the submaxillary gland, although some authors who have not compared the response of this gland with that of other organs have come to such a conclusion.

* These studies were carried out with the collaboration of R Ross.

Conclusions

This brief review of the proteases of salivary gland origin merely points out how fragmentary our knowledge is and how crude some of our methods are. First, our observations, and by this I mean our own and those of others, refer to only a few points in time; second, they deal with one or at most two exocrine organs; and, third, they deal with unphysiological dosages of the hormones. Although work has been done in many laboratories, it is difficult to piece the fragments together into a composite picture of the function and control of the physiological activity of the submaxillary gland.

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REGULATION OF SALIVARY GLAND AMYLASE ACTIVITY*

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Secretory activity by digestive glands is characteristically periodic. This periodicity is distinguishable even in such glands as rat pancreas and human submaxillary and parotid, which exhibit a tendency toward continuous basal secretion. During periods of activity several of the digestive glands discharge relatively large quantities of hydrolytic enzymes into carrier secretory fluid and, perhaps, into the blood. In such cases a relationship between secretory discharge and ensuing reconstitution of secretory protein stores in the gland has frequently been assumed; the clearest exposition of this has been that of Langstroth *et al.*[1]

Langstroth and his co-workers selected the pancreas as their biological experimental system. Here, initial secretion in response to an appropriate stimulating agent involves depletion of preformed gland stores of secretable protein, for example, hydrolytic enzymes and enzyme precursors. Cessation of stimulation leads eventually to replenishment of gland protein stores as a result of synthesis of new protein by the pancreas cells. This replenishment process is unaffected by blockage of parasympathetic influences. Langstroth and his co-workers postulated that intermediate reactions in the synthesis process are reversible. Adherence to the law of mass action would then link synthesis rate to degree of depletion. The assumptions are simple and, in the light of newer work on amino acid activation and incorporation, they are also plausible. Additional information concerning actual synthesis rates at various stages of the secretion cycle is, however, urgently needed.

In contrast to the sequence of events described for the pancreas and characteristic also of the parotid gland—that is, stimulation, depletion and then synthesis—the pattern of active enzyme accumulation in the submaxillary-sublingual gland of the rat is wholly different. Stimulation of this gland, instead of depleting the tissue of amylase, a secretory enzyme, leads to a rapid, progressive, and appreciable rise in gland amylase level. This rise is dependent on continuing parasympathomimetic influence. Evidence indicates that amylase accumulation in the submaxillary-sublingual gland of the rat is the result, as in the pancreas, of synthesis of new enzyme. In the case of the submaxillary-sublingual gland, however, this synthesis is subject to relatively direct regulation by the parasympathetic branch of the autonomic nervous system.

Experimental Methods

Long-Evans rats reared on standard laboratory diet but fasted 12 to 48 hours before experiment were used for all studies. Animals were anesthetized lightly by subcutaneous or intraperitoneal injection of pentobarbital. Blood samples were obtained, without use of anticoagulant, from the jugular vein

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and centrifuged under oil. Amylase was determined on serum and supernatant of gland homogenate by the method of Myers *et al.*,[2] modified slightly for smaller volumes Amylase levels were expressed in terms of milligrams of reducing substance (glucose) formed during a 15-min. digestion period at 37° C. from 1 mg. or gm. wet weight (w. w.) of gland or 1 ml. of serum. Enzyme activity of the homogenate supernatant was compared in phosphate and barbital buffers to evaluate the contribution of phosphorylase activity, which was found negligible under conditions of these experiments. Sodium and potassium were determined on dry-ashed samples by flame photometry.

Results

Amylase Levels in Fasted, Unstimulated Rats

For organs and body fluids that maintain some detectable level of amylase, this level is generally of fairly stable and characteristic magnitude, provided

Table 1
Amylase Levels* of Serum, Salivary Glands and Liver of Unstimulated Fasted Rats

No	Serum	Submaxillary gland	Liver	Parotid gland†
1	48	1 2	16	563
2	44	2 5	17	604
3	68	1 5	31	489
4	53	4 7	16	605
5	48	22 0	21	552

* As mg reducing sugar formed/ml serum or gm wet tissue Corresponding serum, submaxillary, and liver levels were obtained from each of five animals fasted for 48 hr Parotid gland levels, obtained separately with the use of animals fasted 12 hr, were reported previously,[7] but are now corrected for the dilution factor omitted earlier
† Multiply parotid gland levels by 1000

conditions of environment, for example, nutrition[3] or hormonal state[4] and heredity[5] are maintained reasonably constant. Thus, from the data in TABLE 1 and those of Wiberg and Tuba[3] it is apparent that, following a fasting period of moderate duration, amylase levels of selected tissues assume the following progression with relation to serum.

parotid gland > pancreas > serum > liver > submaxillary gland

In the case of liver, amylase level is somewhat too high to be accounted for by amylase of extracellular fluid, as indicated also by the work of McGeachin *et al.*[6] With submaxillary-sublingual gland, however, the tissue level of an appropriately fasted animal frequently can be completely accounted for by the amylase probably present in the vascular system of the gland, although higher levels are observed.

Effect of Stimulation on Amylase Levels of Tissue

Administration of pilocarpine or ad libitum feeding following a fast results in depletion of parotid gland stores of amylase, frequently to an extent of 50

per cent (TABLE 2). Similar depletion is observed with pancreas during stimulation.[8] Few data are avilable from which amylase synthesis rates in the absence of stimulation may be compared to rates of synthesis during stimulation. The two experiments outlined in TABLE 3, however, indicate that the rate of synthesis in the parotid gland during stimulation for 60 or 180 min. is not appreciable. It does not appear, therefore, that the synthesis rate in the parotid gland is enhanced during the stimulation period.

TABLE 2
EFFECT OF STIMULATION *in Vivo* ON THE AMYLASE LEVEL OF PAROTID GLAND OF FASTED RATS

No. of animals	Unstimulated gland (I) · mean amylase level*	Conditions of stimulation	Stimulated gland (II) mean amylase level*
5†	538	Pilocarpine for 6 hours	240
17‡	569	Fed ad libitum	240

* As corrected values[7] for mg reducing sugar/gm. wet gland when multiplied by 1000.
† Gland I was removed, after 12-hour fast, from each animal under light Nembutal anesthesia before stimulation of Gland II was begun.
‡ Paired glands were not used in this case. Amylase levels were determined on 17 fasted animals and on 17 animals fed ad libitum
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TABLE 3
EFFECT OF PILOCARPINE ON TOTAL ACTIVE AMYLASE PRODUCED PER MILLIGRAM OF GLAND TISSUE BY PAROTID GLANDS OF FASTED RATS

Control gland amylase level	Pilocarpine administered*				Increase (%)
	Duration (min)	Secretion (S) amylase level†	Stimulated gland (SG) amlase level	Total S + SG amylase level	
625	180	327	390	717	15
520	60	88	465	553	6

* Pilocarpine was administered subcutaneously (24 mg /hr.) in divided doses
† Amylase levels expressed as mg reducing sugar formed/mg. wet gland[7]
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A different pattern of change in tissue amylase level during pilocarpine stimulation is observed for the submaxillary-sublingual gland. Here, amylase levels increase progressively in orderly fashion with increasing duration of stimulation. This is shown in FIGURE 1, which also shows that this effect is unaccompanied by any appreciable or consistent change in gland w. w. Other observation has shown that submaxillary gland secretion is copious during the period of pilocarpine stimulation, and that amylase has access to the secretory fluid.

Stimulation by feeding, following a 48-hour fast, also results in increased amylase levels in rat submaxillary glands. As shown by the data in TABLE 4, amylase levels may increase fifteenfold during a 2-hour feeding period following the 48-hour fast. Even within 20 min. after the start of feeding, an appreciable rise in gland amylase level is noted. With feeding, as with administration of pilocarpine, there is a progressive increase in gland enzyme level with increasing

duration of stimulation. Similar results are obtained following subcutaneous administration of acetylcholine.[9]

The action of atropine on accumulation of amylase by the parotid gland and by the submaxillary-sublingual gland differs. Administration of atropine to a

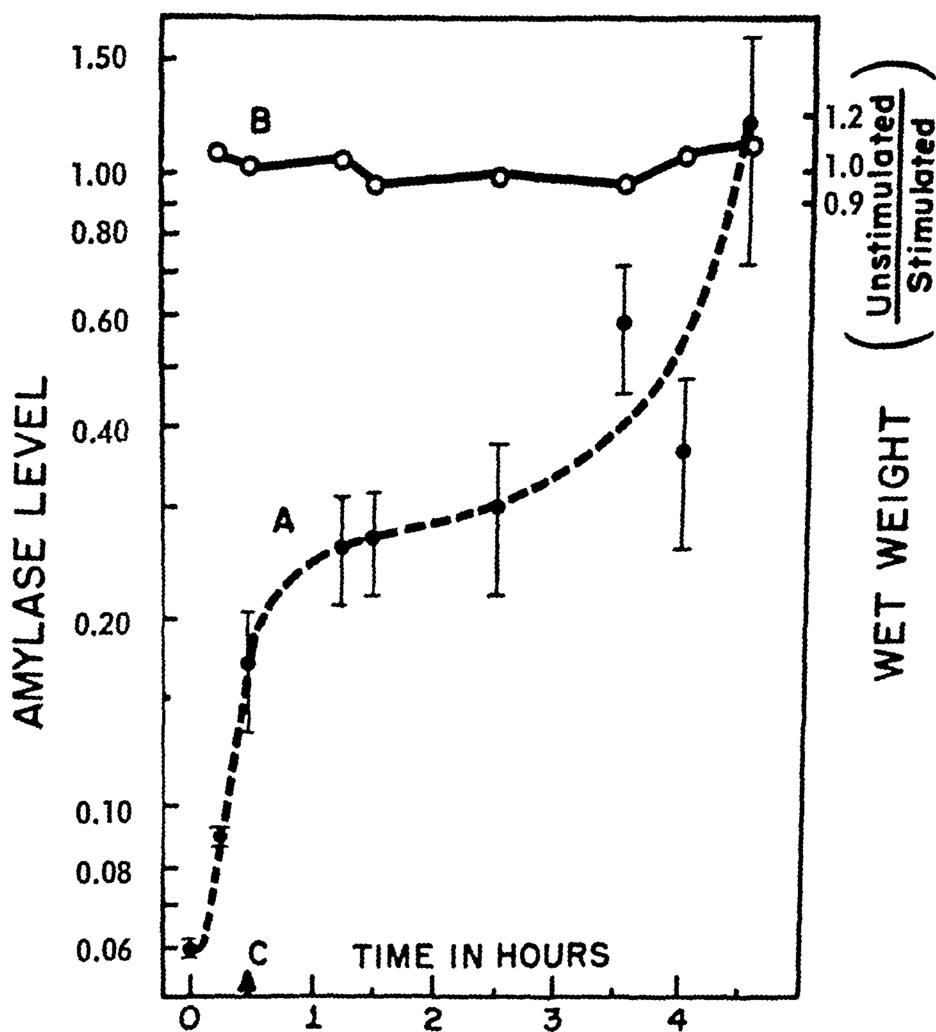


FIGURE 1. Changes in amylase levels in milligrams of reducing sugar per milligram of wet tissue (curve A), and gland wet weights (curve B), during pilocarpine stimulation. The point C shows the effect of atropine administered both 40 min before and during a 30-min period of pilocarpine stimulation Each point beyond zero time represents the mean value for 8 animals, standard errors of the means being indicated by vertical bars. The mean at zero time is that for the control glands of all the subsequently pilocarpinized animals Reproduced by permission from *The American Journal of Physiology*[27]

depleted parotid gland has no effect on amylase accumulation, as shown in TABLE 5. In this experiment parotid glands were depleted by ad libitum feeding; one gland was removed for estimation of amylase level before atropine administration; atropine was administered periodically over 8 hours; and, finally, the second parotid gland was removed for amylase analysis. Accumulation of amylase to the extent of 42 per cent of the initial level was observed.

Accumulation to a similar extent occurred when the experiment was performed without administration of atropine.

With the submaxillary-sublingual gland, administration of atropine during the period of stimulation by pilocarpine, for example, completely suppresses amylase accumulation (FIGURE 1). Similar inhibition of the accumulation effect has been observed when atropine is administered during stimulation by feeding (TABLE 4) or acetylcholine.[9]

Enhancement of amylase levels in the parotid gland or the pancreas after cessation of parasympathomimetic stimulation is attributable to the synthesis of new enzyme Three general mechanisms that could account for the observed increase in amylase level in the submaxillary gland during stimulation are (1) activation of a proenzyme or bound (that is, "masked") enzyme form, (2) increased serum space or serum amylase level, and (3) synthesis of new enzyme by submaxillary-sublingual gland tissue cells.

TABLE 4
EFFECT OF FEEDING ON RAT SUBMAXILLARY GLAND AMYLASE LEVELS

Feeding period (min)	No of animals	Mean food consumption (gm /animal)	Atropine (mg /rat)	Amylase level* (M ± S E)
0	13	0		0 02 ± 0 003
20	10	2 1		0.19 ± 0 078
60	10	3 9		0 16 ± 0 035‡
120	10	4.3		0.29 ± 0 83‡
60	10	0 8†		0 08 ± 0 018‡
60	9	0 8	70	0 01 ± 0.004

* As mg reducing sugar/mg wet tissue; M ± S E · mean ± standard error
† $p < 0\ 002$ (value after 20 min · $p < 0\ 02$).
‡ Amount of food limited to that ingested by atropinized group in 60 min [9]
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TABLE 5
EFFECT OF ATROPINE ON AMYLASE SYNTHESIS IN PAROTID GLAND

	Percentage increase in amylase level* M ± S E
Control (9)†	38 ± 11
Atropine (6)	42 ± 6

* Eight hours postprandial
† The number of animals is indicated in parentheses

Effects of Chemical and Physical Agents in Vitro on Gland Amylase Levels

To determine whether the activation of an enzyme precursor form can account for the increased amylase level of stimulated submaxillary gland, a group of experiments involving primarily the use of gland homogenates and slices was undertaken. Homogenates treated with sodium oleate, trypsin, urea, heat, *n*-butanol, or hydrogen ion, all of which are agents known to activate

precursors of other enzymes,[10-13] or with pilocarpine, failed to show a significant increase in amylase levels (TABLE 6). Treatment of substrate-free amylase with each of these agents caused no appreciable inhibition, except in the case of *n*-butanol. In addition, the amylase level of homogenate of unstimulated submaxillary gland does not change following treatment with extract of gland previously stimulated *in vivo*. Finally, pilocarpine does not cause an increase in the amylase level of submaxillary gland slices in an oxygenated but otherwise unfortified medium, and addition of amylase to the medium, in the presence or absence of pilocarpine, does not result in an increase in amylase levels. Therefore, it does not appear that a release of free enzyme from a precursor or bound state is the mechanism of the increase in amylase activity which occurs *in vivo* following parasympathomimetic stimulation

TABLE 6
EFFECTS OF CHEMICAL AGENTS ON AMYLASE LEVELS OF RAT SUBMAXILLARY GLAND HOMOGENATES

Chemical agent	Concentration	Duration* (min)	Amylase level†	
			Control gland	Treated gland
Sodium oleate	0 04%	30	0 02	0 02
Urea	4 M	30	0 02	0 03
n-Butanol	0 76 M	30	0 0	0 03
Trypsin	1 mg -%	30	0 01	0 02
H^+	*p*H 4 5	15	0 01	0 0
	*p*H 9 5	15	0 01	0 0
Pilocarpine	0 2 mg /ml.	90	0.02	0 03
Homogenate of stimulated submaxillary	1·2	30	0.58‡	0 62

* At 37° C
† As mg reducing sugar/mg wet gland
‡ Amylase level is the arithmetic sum of the amylase level of the control gland and added homogenate of stimulated gland.

Gland Amylase Levels in Relation to Serum Amylase

Changes in the vascular bed occurring during stimulation of the submaxillary-sublingual gland do not appear to be the prime cause of the large increase in gland amylase that accompanies this stimulation. Evidence in this regard is provided by data (FIGURE 1 and TABLE 4) that show that simultaneous administration of atropine suppresses any effect of parasympathomimetic stimulation on gland amylase level although, as repeatedly demonstrated, atropine is not effective in blocking the vasodilation accompanying such stimulation.[14,15]

Although vascular changes cannot account for enzyme changes in the submaxillary-sublingual gland of the magnitude encountered, the possible relationship of gland enzyme levels to increases in serum amylase during stimulation has required further exploration. Antopol and his co-workers[16-18] have shown that administration of acetyl-beta-methylcholine to dogs or rabbits results in a marked progressive rise in serum amylase during an ensuing period of 3 to 6 hours. This increase in serum amylase is inhibited by atropine and seems attributable to loss to the blood of pancreatic enzyme during secretion.[17]

Amylase levels of serum, submaxillary-sublingual gland and, for purposes of comparison, liver, have been determined for 5 fasted, unstimulated rats and for 8 animals similarly fasted and administered pilocarpine for 4 hours, as shown in TABLE 7. The amylase level in livers of unstimulated animals is lower than that of serum but, as indicated by liver/serum amylase ratios, it is probably somewhat too high to be accounted for entirely by amylase of extracellular space. Extracellular space of rat liver has been estimated to be approximately 20 per cent of total organ space.[19] The presence of some intracellular amylase in liver has been suggested recently by McGeachin *et al.*[6] In the absence of stimulation, submaxillary gland/serum amylase ratios may be even lower than

TABLE 7

EFFECT OF PILOCARPINE ON AMYLASE LEVELS OF RAT TISSUES AND SERUM

Duration of stimulation (min)	Amylase levels, mg glucose/gm w w.				
	Serum	Liver	Liver/serum	Submaxillary	Submax / serum
0	47 5	16 1	0 34	1.24	0 026
	43 8	16.9	0 39	2.52	0 058
	67 5	30 5	0 45	1.52	0.023
	52.5	16 3	0.31	4 69	0 089
	47.5	20 5	0 43	22 0	0 46
	339*			24.0	0 071
240	255	50 0	0 20	725	2.8
	315	70 0	0 22	1550	4 9
	966	97.0	0.10	4305	4 5
	521	46 3	0.09	1615	3 1
	546	57 5	0.11	3090	5 7
	574	84 8	0 15	2530	4.4
	421			1388	3 3
	266			471	1 8

* Serum amylase elevated by I V injection of parotid gland extract after removal of control submaxillary gland and ligation of renal pedicles of recipient Blood sample and second submaxillary removed 2 hours later. Control submaxillary showed negligible amylase activity

liver/serum ratios and frequently of an order of magnitude that suggests absence of amylase within the tissue cells of unstimulated submaxillary gland.

Administration of pilocarpine over a 4-hour period results in a marked rise in serum amylase level and in a lesser rise in liver amylase level. The liver/serum ratio declines from a value of 0.3 to 0.4 in the unstimulated state to approximately 0 1 to 0.2, after 4 hours of pilocarpine administration. From the failure of the liver/serum ratio to rise, it is at least clear that appreciable accumulation of amylase does not occur in liver as a result of administration of the parasympathomimetic agent and ensuing increased serum amylase level. The actual reduction in ratio may be due to the overwhelming effect of the now-preponderant extracellular fraction of amylase.

Unlike the liver amylase level, the submaxillary-sublingual gland level after pilocarpine administration rises appreciably more than does the serum level, and stimulated submaxillary/serum ratios ranging up to 5.7 have been ob-

served. This value represents a greater than twentyfold increase in submaxillary-sublingual amylase over the serum amylase contributed by extracellular space (25 per cent; Schneyer and Schneyer, unpublished) even if this space is assumed to be freely accessible to the enzyme. It is, moreover, quite unlikely that serum amylase enters the tissue cells to any appreciable extent[20] Additional data in TABLE 7 indicate that the submaxillary gland, at least in the unstimulated state, does not accumulate amylase in the face of a serum level elevated by intravenous injection of the enzyme.

Normal feeding after a 48-hour fast also results in an increase in serum and submaxillary gland amylase, as shown in TABLE 8. Submaxillary gland/serum amylase level ratios in each case indicate gland levels in excess of those accountable for by extracellular space amylase, and ratios greater than unity are observed.

TABLE 8

EFFECT OF FEEDING ON AMYLASE LEVELS OF RAT SUBMAXILLARY GLAND AND SERUM

Duration of feeding period (min)	Amylase levels*		
	Serum	Submaxillary	Submax./serum
90 (5.6)†	185	110	0 60
(5 4)	160	130	0 81‡
140 (5.6)	125	55	0 44
(1 2)	110	148	1.35
160 (7 4)	215	375	1 74
(6 4)	105	55	0 52

* Mg reducing sugar/gm w w.
† Numbers in parentheses indicate grams of food ingested during feeding period.
‡ Pilocarpine stimulation after removal of this gland and blood sample resulted, 90 min later, in an amylase level in serum of 170, in submaxillary, 275; the submaxillary/serum ratio rose to 1 62

The recent suggestion from the work of McGeachin and Lewis[21] that normal human serum contains an amylase inhibitor not present in pancreatitis serum has been explored in relation to amylase increases observed in rat serum and submaxillary gland during stimulation. It has been shown already (TABLE 6) that pilocarpine does not enhance the amylase levels of submaxillary gland homogenate Pilocarpine *in vitro* also fails to increase rat serum amylase activity during 60-min. contact at room temperature. Moreover, it does not appear (TABLE 9) that the serum of stimulated animals contains a counter-inhibitory factor capable of unmasking inactive amylase of unstimulated gland, since the addition of activated serum to homogenate of unstimulated gland does not raise amylase activity of the mixture.

Therefore, extensive investigation has failed thus far to demonstrate that the accumulation of amylase in the submaxillary-sublingual gland during parasympathomimetic stimulation is the result of activation of an enzyme precursor form. Similarly, concomitant increases in serum amylase have been shown to bear no prime relationship to gland increases. Accumulation of active enzyme

by the submaxillary-sublingual gland during parasympathomimetic stimulation is interpreted, therefore, as resulting from synthesis of new enzyme.

Electrolyte Changes in Glands During Activity

In view of the well-known regulatory effects of electrolytes on other physiological activities and the recent evidence of influence *in vitro* of sodium-potassium balance on several processes[22,23] which are considered an integral part of the over-all protein synthesis reaction, investigation was made of sodium and potassium levels of unstimulated salivary glands and of glands stimulated by pilocarpine or normal feeding. Data are shown in TABLE 10. It is evident that the salivary glands of the rat fall into two groups with regard to sodium-

TABLE 9

AMYLASE LEVELS OF SERUM FROM PILOCARPINE-STIMULATED RATS AFTER INCUBATION WITH UNSTIMULATED SUBMAXILLARY-SUBLINGUAL GLAND HOMOGENATE

Amylase level, mg glucose/gm serum			Contribution by gland
Undiluted serum	Serum diluted 1 1 3 (calculated)	Serum* diluted 1 1 3 (by gland homog)	
413	317	313	0
266	205	185	0

* Incubated 1 hour at 25° C Amylase level of serum alone showed no decrease. Amylase activity of unstimulated control glands is virtually undetectable at dilution (*ca* 1:100) used for serum analysis

TABLE 10

EFFECT OF PILOCARPINE STIMULATION ON WATER AND ELECTROLYTE CONTENT OF RAT SUBMAXILLARY, SUBLINGUAL, AND PAROTID GLANDS*

	Unstimulated M ± S E	Stimulated M ± S E	Significance of difference (p)
A Submaxillary gland	15 Animals	12 Animals	
Water/dry	3 1 ± 0 062	2 8 ± 0 051	0 0000
Na	40 7 ± 1 74	46 6 ± 2 08	0 0139
K	92 1 ± 1 60	75 2 ± 2 41	0.0000
Na/K	0 44 ± 0 019	0 63 ± 0 033	0 0000
B Sublingual gland	10 Animals	10 Animals	
Water/dry	3 4 ± 0 090	3 5 ± 0 084	
Na	43 2 ± 1.87	54.8 ± 2 67	0 0009
K	83 1 ± 2 02	71.6 ± 1 62	0 0001
Na/K	0 52 ± 0 023	0.77 ± 0 034	0 0000
C Parotid gland	15 Animals	11 Animals	
Water/dry	2 5 ± 0 059	2.5 ± 0 078	
Na	60 7 ± 2 46	48 4 ± 1 87	0 0001
K	65 4 ± 1 52	63 6 ± 2.25	
Na/K	0 94 ± 0 051	0.77 ± 0.036	0.0035

* The glands were stimulated for 20 min Electrolytes are expressed as mEq /kg wet weight [24]

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potassium balance in the fasting, rested state and the direction of change in that balance during stimulation by pilocarpine for a period of 20 min. Submaxillary and sublingual glands of unstimulated, fasted animals contain relatively high levels of potassium and low levels of sodium. Stimulation of these glands results in a loss of potassium, gain of sodium, and a rise in Na/K ratio. These effects are in agreement with reported observations[25,26] on cat and dog submaxillary glands.

The parotid gland of the rat, on the other hand, behaves in a different manner. Resting parotid gland of fasted animals contains a relatively high level of sodium. During activity, sodium, not potassium, is lost, and the Na/K ratio declines from a resting level appreciably higher than that for submaxillary and sublingual glands to a value more closely approximating, or even identical to, the level for active submaxillary and sublingual glands.

In rat submaxillary glands, accumulation of amylase can be detected generally within the first 20 to 30 min. of stimulation. In the parotid gland, although the time rate of accumulation is uncertain, the extent of accumulation is apparently not appreciable even after 180 min. of stimulation. Accordingly, an experiment was designed to determine the comparative duration of electrolyte change with continued stimulation of the two gland types. Continued ad libitum feeding was used as stimulation. Submaxillary and sublingual gland sodium and potassium levels and Na/K ratios did not differ appreciably, after the long continued stimulation, from fasted resting levels (TABLE 11). Under the same conditions, however, parotid gland values were significantly different from values for resting glands, generally following the pattern characteristic for short-term stimulation of the gland.

TABLE 11

EFFECT OF STIMULATION BY AD LIBITUM FEEDING ON WATER AND ELECTROLYTE CONTENT OF RAT SALIVARY GLAND*

	Submaxillary M ± S E	Sublingual M ± S E	Parotid M ± S E	Parotid p†
Water/dry	3 0 ± 0 057	3 7 ± 0 063	2 8 ± 0 075	0 0002
Na	33 7 ± 1 19	41 3 ± 2 12	53 6 ± 1 43	0.0107
K	88 3 ± 2 26	87 7 ± 1 88	75 8 ± 1 50	0 0000
Na/K	0 38 ± 0 018	0 48 ± 0 031	0.71 ± 0 026	0 0000

* Submaxillary and sublingual glands obtained from 10 animals, parotid glands from 12 animals [24]

† Significance of difference of stimulated from unstimulated parotid levels

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In terms of possible correlation of sodium-potassium balance to gland activity involving enzyme formation, these electrolyte changes appear consistent. Thus, with stimulation for brief periods, which would, however, encompass the beginning of acceleration of synthesis in submaxillary and sublingual glands, Na/K ratios of all glands approach a similar value; only in the case of parotid gland, where acceleration of synthesis probably is delayed, does this new Na/K ratio persist with continuing stimulation.

Discussion and Summary

These investigations are concerned with factors of possible functional importance in the regulation of accumulation of secretable hydrolytic enzymes by digestive glands. The parotid gland in the rat displays a pattern of depletion and accumulation similar to that generally described for the pancreas. The accumulation results from synthesis of new enzyme and is dependent on prior depletion of enzyme stores. Parasympathetic blocking agents are without effect on this accumulation process. The autonomic nervous system, therefore, does not regulate directly the process of amylase synthesis in the parotid gland.

The submaxillary-sublingual gland has been shown in these investigations to display a different pattern of regulation of active enzyme accumulation. In this gland complex, parasympathomimetic activity causes no initial depletion of amylase, the gland digestive enzyme, but leads instead to an appreciable, rapid, and progressive increase in gland enzyme level. This increase is inhibited by atropine and apparently is not attributable to activation of an amylase precursor, release of amylase from binding sites, unmasking of an inactive form of the enzyme, or to a simultaneously occurring rise in serum amylase level. The enzyme accumulation in the submaxillary-sublingual gland is best explained by assuming a synthesis of new enzyme, in this case under the control of the parasympathetic nervous system. Electrolyte studies indicate that the Na/K balance of the tissues, although different for the two gland types in the resting state, is similar during the acceleration of the synthesis process.

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AEROBIC OXIDATIVE METABOLISM OF SALIVARY GLANDS

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The concept that respiratory processes provide the energy required for salivary gland secretion can be traced back to 1886, when Chauveau and Kaufmann first reported an increased oxygen consumption by the secreting parotid gland.[1] Since then, numerous experiments have established the existence of a positive relation between salivary secretion and aerobic oxidation. Earlier experiments performed, for the most part, with glands stimulated to greater secretion indicated that the increase in secretory activity was paralleled by a corresponding increase in oxygen consumption. Such studies showed that chemical or electric stimulation of salivary secretion *in vivo* by intact submaxillary glands produced a significant increase in glandular oxygen uptake.[2-4] A similar increase in oxygen utilization was shown to occur *in vitro* when salivary tissue slices were exposed to secretory stimulants, such as acetylcholine, eserine, and pilocarpine, whereas sections incubated anaerobically under identical conditions did not respond.[5,6]

More recently, a converse approach, based upon the use of nonsecreting glands, confirmed the previously established relationship between oxygen utilization and salivary secretion. By ligating the submaxillary excretory duct, Junqueira and his co-workers[27] produced submaxillary glands in a so-called resting condition in which the glands, while not secreting, remained capable of secreting upon adequate stimulation. Such nonsecreting glands had a significantly lower oxygen consumption and a lower high-energy phosphorus content than did the nonligated controls, anaerobic glycolysis again remained unaffected by the secretory state of the gland.[7,8]

Additional information bearing on the role of oxidative processes in salivary functions comes from recent studies demonstrating that glandular alterations caused by severe X irradiation and acute starvation are accompanied by disturbances in salivary gland aerobic intermediary carbohydrate metabolism.[9,10] Moreover, iodide concentration and iodotyrosine synthesis by submaxillary homogenates both have been shown to be mediated by aerobic oxidative reactions involving an iodide peroxidase.[11] Finally, a current report reveals that salivary glands possess an unusually high monoamine oxidase activity.[12]

The present study was prompted by the need for additional relevant data that will lead to an increased understanding of the oxidative processes operating in salivary glands Our immediate objective was a comparison of the aerobic oxidative capacities of the two main types of epithelial structures comprising the salivary gland parenchyma, that is, acini and ducts.* The two approaches employed were (1) a conventional histochemical technique that permits the visual comparison of oxidase activity in terms of the intensity of the indophenol blue reaction in frozen tissue sections and (2) an original differential centrifugation procedure that permits the quantitative estimation of cytochrome oxidase activity in isolated acini and in isolated ducts.

* For a clarification of the use of the term ducts see the first paragraph under *Discussion*.

Methods

The histochemical study was made with the use of the classic Nadi reaction[13,14] on frozen sections of rat submaxillary, parotid, and sublingual glands. Color estimation of indophenol blue formation was made after a three-to-five-minute interval. Cyanide and azide controls were used as previously described.[15]

The isolation procedure that yields rabbit submaxillary gland acini and ducts in separate fractions was developed with a medium of 70 per cent glycerol.[16,17] As a preliminary communication, this report will outline the method only briefly, since full details and discussion of the procedure necessitate a more elaborate presentation.[18] Several grams of fresh tissue are crushed through bolting cloth to release intact acini, which are then sedimented and washed centrifugally. The residue of ducts and unbroken tissue on the filter cloth is homogenized at high speed to disintegrate residual acini. The homogenate is then filtered, and the filtrate centrifuged for sedimentation and washing of the duct segments. Following isolation of these structures, the disruption of their cell membranes is accomplished by homogenizing at specific speeds predetermined for acini and for ducts.

For the quantitative enzyme analysis, cytochrome oxidase activity was assayed in accordance with the scheme of Wainio,[19] where the activity is expressed in micromoles of cytochrome *c* oxidized per minute per milligram of protein. Protein determinations were made by the method of Lowry *et al.*[20]

Results

In the indophenol blue histochemical reaction of the three rat salivary glands studied, the ducts consistently and reproducibly revealed an intense indophenol blue synthesis, indicative of marked aerobic oxidative activity, whereas the acini in all cases were much less active. The greatest activity occurred in the ducts of the sublingual gland, whereas those of the submaxillary and parotid glands showed fairly comparable intensities of a slightly lower order of magnitude. FIGURE 1 shows a typical relationship between indophenol blue synthesis in ducts and in acini of the submaxillary gland, and is representative of the salivary glands studied. The greater activity of duct structures is evident. The intensities of black appearing in the photomicrograph represent fairly accurately the intensities of blue appearing in the original tissue section. The cyanide and azide controls gave completely negative results.

As in the histochemical study on rat glands, spectrophotometric assays of cytochrome oxidase in rabbit glands consistently indicated higher activities in ducts than in acini. TABLE 1 lists the data obtained for the cytochrome oxidase assay of the isolated acini and ducts. The ratio of cytochrome activity of ducts to that of acini is repeatedly greater than 1 and reaches a maximal value of 1.90.

The rabbit submaxillary acini isolated in 70 per cent glycerol were intact and morphologically similar to those in frozen tissue sections examined under the same magnification. FIGURE 2 shows some isolated acini, with their typical arrangement as anatomic aggregates. An enlarged view of a selected field in FIGURE 3 emphasizes the pyramid-shaped cells with basal nuclei and intact intercellular membranes.

The rabbit submaxillary ducts were isolated as duct segments. FIGURE 4 shows segments of these structures recovered as both elongated and transverse fragments. No attempt was made to identify the different types of segments

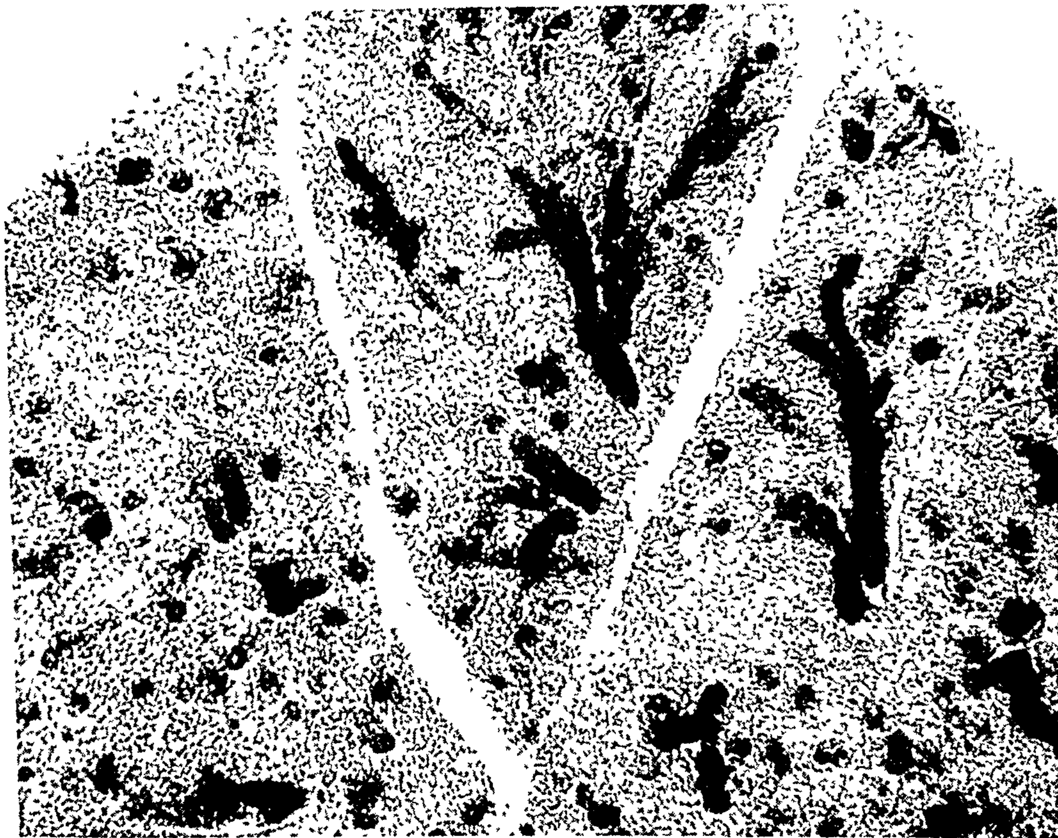


FIGURE 1 Indophenol blue synthesis by fresh frozen section (40 μ) of rat submaxillary gland Note the greater activity of duct structures than that of acini.

TABLE 1
CYTOCHROME OXIDASE ACTIVITIES OF HOMOGENATES OF ISOLATED DUCTS AND OF ISOLATED ACINI
Activities Expressed as μM Cytochrome c Oxidized/mg Protein/Minute

Ducts	Acini	Ratio of ducts acini
0 0672	0 0589	1.14
0 0714	0 0610	1 17
0 0797	0 0605	1 23
0 0887	0 0694	1.28
0 0565	0 0297	1 90

obtained, but the size and form of many of the segments shown in FIGURE 5 are highly suggestive of striated ducts.

Discussion

Unlike many other organs commonly employed for the study of cell chemistry, salivary glands, as do exocrine glands, provide the biochemist with a natu-

ral arrangement of specific cell groups held together as anatomic aggregates. The parenchyma of the three major mammalian salivary glands, that is, submaxillary, parotid, and sublingual glands, consists of two main types of epithelial aggregates, in the form of acini and ducts. Each acinus represents a cluster of epithelial cells held together in a unit as a compact mass. The complexity



FIGURE 2. Isolated acini, rabbit submaxillary gland Wet stain methyl green-pyronin ×250



FIGURE 3 Isolated acini, rabbit submaxillary gland Wet stain methyl green-pyronin. Oil immersion. ×1000.

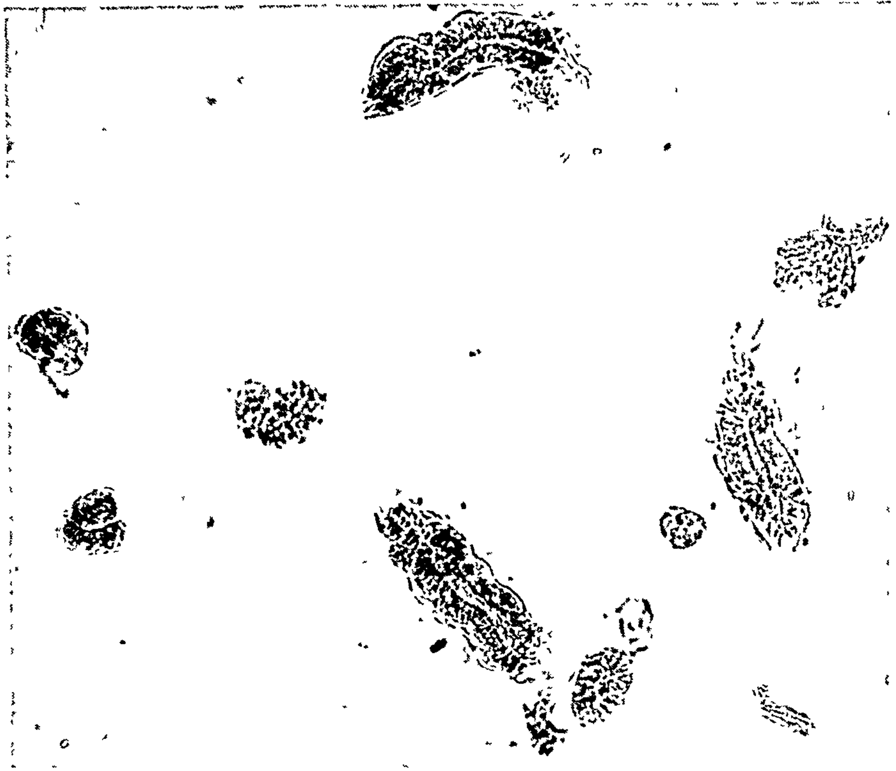


FIGURE 4. Isolated duct structures, rabbit submaxillary gland. Unfixed, unstained. ×100.



FIGURE 5 Isolated duct structures, rabbit submaxillary gland. Unfixed, unstained. Dark phase contrast ×100.

of the duct system varies with the glands considered, but is conceded, in the case of the rat, to be greatest in the submaxillary gland. Here several divisions of a continuous intralobular duct unit are identifiable from specific groups of epithelial cells, constituting, in effect, several types of aggregates. Leading from the acinus, a branching flat-celled intercalated duct, which represents a second type of epithelial aggregate, unites the acinar mass with a third type of aggregate, the granular secretory tubule, which in turn is continuous with a fourth type of aggregate, the striated excretory duct.[21] Anatomically, therefore, as well as physiologically, the duct may be considered as a triple composite of aggregates, naturally differentiated with respect to its proximal intercalated, intermediate secretory, and distal collecting portions. Except where otherwise indicated, the term "duct" in this report is used *nonspecifically* to denote any or all three portions of the continuous intralobular portion of the duct unit leading distally from the acinus, without distinguishing between the morphologic segmentations discussed above.

Rat salivary glands were used for the histochemical phase of the investigation, in the hope of building upon the considerable cytological information currently available concerning them. The histochemical finding, with the use of tissue slices, that oxidative activity exists in both ducts and acini, in strikingly different proportions, suggested the desirability of recovering these aggregates in quantity in order to permit further biochemical analysis of their oxidative capacities on a quantitative scale. Of the three rat salivary glands examined, the submaxillary gland seemed most promising as working material because of its greater mass and its freedom from fat. Unfortunately, however, considerable difficulty was experienced in releasing acini from rat glands, which necessitated the ultimate abandonment of the use of the rat. The rabbit submaxillary gland proved to be more favorable for fractionation, from the standpoints both of recovery and of manipulative convenience, and was therefore used as source material for the isolation procedure.

The procedure finally developed for the successful isolation of anatomic aggregates of epithelial cells, as separate active fractions, combines the principles of differential homogenization and differential centrifugation. Fortunately, there exists in the rabbit submaxillary gland a twofold difference in fragility between acini and ducts, which permits the separation of these two types of epithelial aggregates on the basis of their response to homogenization treatment. The acini, which are more delicate than the ducts, are removed by gentle crushing and filtration More resistant acinar masses adhering to the ducts are disintegrated by homogenization at a speed that leaves the ducts relatively intact. This homogenizing speed equals approximately half of that required for the disruption of ducts Subsequent sedimentation of the acini, which are less dense than the duct segments, requires a higher centrifugal acceleration than do the duct segments.

The finding, obtained by means of two different types of assays, that respiratory activity is higher in the ducts than in the acini is of considerable interest. Similar indications of superior duct activity have been reported for the enzymes succinic dehydrogenase,[22,23] esterase,[24,25] and acid phosphatase.[12,27] The physiological significance of this heightened metabolic activity is yet to be determined.

Summary

Histochemical estimation of indophenol oxidase, as well as quantitative determination of cytochrome oxidase, reveals a higher oxidase capacity for the ducts of submaxillary glands than for their acini.

A preliminary report is made on the method employed for the separation of acini from ducts of the rabbit submaxillary glands in measurable quantities. This procedure introduces a new type of tissue isolate, in the form of anatomic aggregates of similar cells, as they exist in salivary glands.

The ducts are found to be more resistant than the acini to the differential homogenization treatment applied, and this difference in fragility has served to facilitate their separation from acini.

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IODINE METABOLISM OF SALIVARY GLANDS

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It has been known for many years that the salivary glands secrete iodide in the saliva at a concentration higher than in the plasma (Elmer, 1938). Using radioiodide, Schiff *et al.* (1947) showed that at physiological concentrations of iodide, the saliva/plasma iodide concentration ratio (S/P ratio) for the mixed saliva of humans is usually above 20 and may be as high as 100.

Chromatographic analysis of the saliva or salivary glands has shown that in mice (Fletcher *et al.*, 1956), in hamsters (Logothetopoulos and Myant, 1956*a*), and in humans (Cohen and Myant, 1959), all the iodine freshly concentrated from the plasma is in the form of iodide. Though Fawcett and Kirkwood (1954) have shown that homogenates of rat submaxillary gland synthesize monoiodotyrosine (MIT), this synthesis cannot be a necessary step in the transport of iodide from the plasma into the saliva, since thiouracil inhibits MIT synthesis but has no effects on the iodide-concentrating mechanism. In any case, it is not certain that the synthesis observed by Fawcett and Kirkwood occurs in the living animal, since it occurs *in vitro* only in the presence of a high concentration of copper ions. Taurog *et al.* (1957) have found a radioactive substance, probably an oxidation product of iodide, in homogenates of rat submaxillary gland incubated with radioiodide. However, this substance cannot play a part in iodide transport since it is not formed in mouse submaxillary glands, which concentrate iodide, and its formation in rat salivary glands is inhibited by thiouracil. It has been suggested that the salivary glands play a special role in the degradation of diiodotyrosine and thyroxine (Fawcett and Kirkwood, 1954), but this is most unlikely since both substances are metabolized equally well in the presence or absence of the salivary glands (Tong *et al.*, 1955; Myant, 1956). It seems, therefore, that in most species the transport of iodide into the saliva is the primary, if not the only, pathway for metabolism of iodine in the salivary glands.

Apart from the importance of the iodide-concentrating mechanism to the problem of the active transport of anions in general, this mechanism has aroused interest because of its similarity to the first step in the synthesis of thyroxine in the thyroid gland Iodide is known to be concentrated in the thyroid before taking part in the iodination of tyrosine to form the primary components of the thyroxine molecule. In humans, the thyroid/plasma iodide concentration ratio in the presence of thiouracil, which blocks hormone synthesis but not iodide accumulation, is about the same as the S/P ratio for the mixed saliva. In both the thyroid and the salivary glands iodide accumulation is inhibited by perchlorate, thiocyanate, and nitrate. Moreover, Edwards *et al.* (1954) have shown that the order of effectiveness of these anions is the same in salivary glands and in the thyroid. Also, it seems likely that in both glands the inhibition is due to competition between iodide and the inhibiting anion for a common transporting process. The evidence for this is that thiocyanate (Crandall and Anderson, 1934) and perchlorate (Edwards

et al., 1954) have been shown to be concentrated by salivary glands and that thiocyanate has been shown to be concentrated by the thyroid (Logothetopoulos and Myant, 1956*b*).

From all this evidence it is difficult to avoid the conclusion that the mechanism responsible for concentrating iodide in the salivary glands is the same as that in the thyroid. The only observation casting doubt on this conclusion is that thyrotrophic hormone (TSH) apparently does not act on the salivary glands, whereas in the thyroid it is known to stimulate the iodide-concentrating mechanism and to induce hyperplasia. In some unpublished work (Myant),

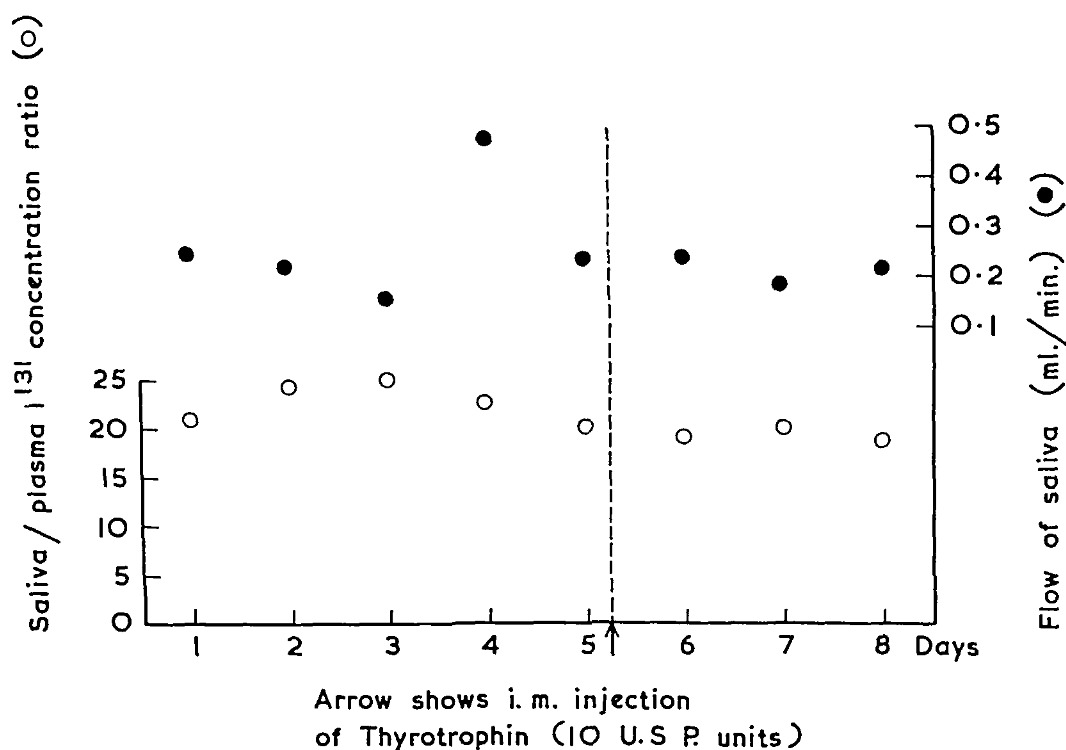


FIGURE 1. Secretion of iodide in human saliva before and after intramuscular injection of thyrotrophic hormone The injected material came from a biologically active batch, as shown by its effect on uptake of I^{131} by the thyroid in humans

injections of TSH into humans were observed to have no effect on the secretion of iodide in the mixed saliva Observations on the S/P ratio were made on three patients during the second week after they had been given about 100 mc. of I^{131} for treatment of thyroid cancer. FIGURE 1 shows the results obtained in 1 patient. The S/P ratio for the mixed saliva and the rate of flow of saliva induced by chewing were measured daily at the same time of day. On the fifth day, 10 U.S.P. units of TSH* were given by intramuscular injection, and the measurements were repeated daily for 3 more days Although the S/P ratio and the rate of flow of saliva varied from day to day, the results show clearly that neither was influenced by the injection of TSH. Similar results were obtained from the other 2 patients.

* Armour Laboratories, Chicago, Ill.

Leblond and Grad (1948) observed atrophy of the secreting tubules of the rat's submaxillary gland after thyroidectomy. This is contrary to what would be expected if TSH stimulates the salivary glands, since thyroidectomy causes an increase in the secretion of TSH. In rabbits, an increase in the secretion of endogenous TSH has no detectable effect on the weights of the salivary glands, as shown in a series of experiments on young adult rabbits (Myant, unpublished) in which the submaxillary glands were removed and weighed three weeks after removal of the thyroid. Although TSH secretion was increased, as shown by the histological appearance of the pituitaries, the weights of the submaxillary glands did not differ significantly from those of litter mates with intact thyroids.

In spite of this negative evidence, the failure of TSH to stimulate the salivary glands is not inconsistent with the hypothesis that the accumulation of iodide is brought about by the same mechanism in the thyroid and the salivary glands. In the salivary glands, the mechanism may be inaccessible to TSH. Again, TSH might influence iodide transport in the thyroid by altering the amount or the availability of some substance which limits the rate at which iodide can be accumulated. There is no reason to assume that iodide accumulation is limited in the salivary glands by the same factors that limit it in the thyroid.

The power to concentrate iodide is present in a wide variety of tissues, as well as in the thyroid and the salivary glands. It is present in the stomach (Elmer, 1938), in the small intestine (Pastan, 1957), in the lactating mammary gland (Honour *et al*, 1952), in the placenta (Logothetopoulos and Scott, 1956), and in the mucous glands of the soft palate of cats and dogs (Cohen and Myant, 1959). In this connection, it is also worth noting that the concentrating power of the salivary glands varies considerably with species and with glands in the same species. In rats, for instance, none of the salivary glands concentrates iodide; in mice and hamsters, in which the submaxillary glands possess a very active concentrating mechanism, the sublingual glands do not concentrate iodide. In each of these tissues in which the effect of thiocyanate has been investigated, it has been found that thiocyanate inhibits accumulation of iodide at low concentrations which have no measurable effect on other metabolic processes. This suggests that all the tissues mentioned above, despite differences in their origin and histological structure, share with the thyroid and salivary glands a common mechanism for concentrating iodide. If this possibility is accepted, the distribution of the mechanism must be taken into consideration in any attempt to understand how the salivary glands concentrate iodide.

One approach to this problem is to try to identify the cells of the salivary gland in which the concentrating mechanism is present. A limited amount of information has been gained by means of autoradiography. Serial autoradiographs (FIGURE 2) of the submaxillary glands of mice and hamsters injected with radioiodide show selective blackening over the secretory ducts but never over the acini (Logothetopoulos and Myant, 1956*a*). The concentrating mechanism must therefore be located in the rodded cells lining the secretory ducts and not in the acini. This raises the possibility that iodide is concentrated in the saliva by the duct cells which would reabsorb water and

electrolytes other than iodide from a primary fluid secreted by the acini. The hypothesis that the ducts modify a fluid derived from the acini is supported by the experiments of Burgen and Seeman (1958) on potassium secretion in dog saliva. However, Fletcher *et al.* (1956) have shown that slices of mouse submaxillary gland, when incubated in a buffer, concentrate iodide from the surrounding medium. It is difficult to see how this could take place if iodide

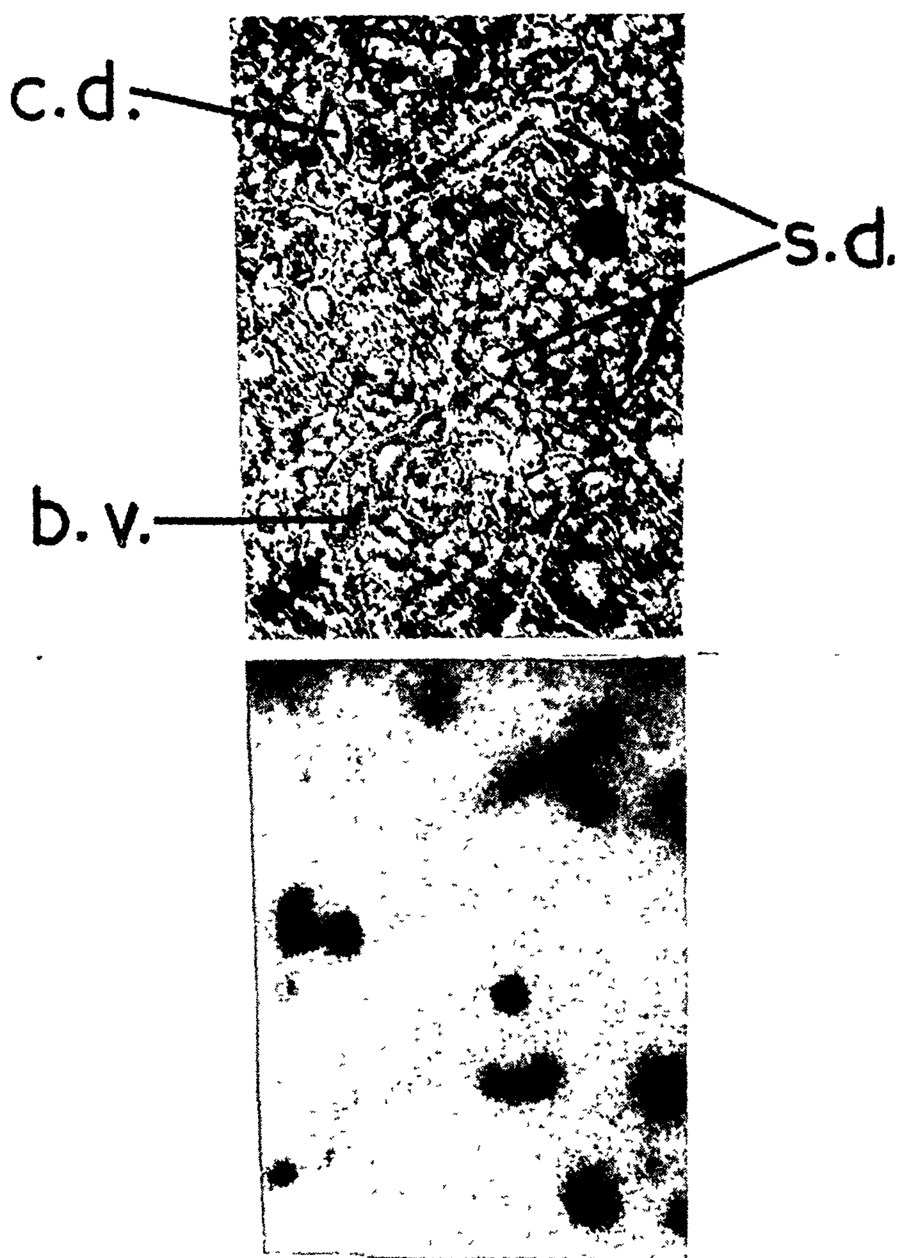


FIGURE 2. Contact autoradiograph of a section of hamster submaxillary gland removed from the animal 1 hour after injection of radioiodide The pattern of blackening corresponds to the distribution of the secretory ducts (s.d) The intensity of blackening over the acini and over some of the collecting ducts (c d) is no greater than that over the blood vessels (b v) Owing to the low resolving power of the method, it is not possible to say whether the blackening is more intense over the lumen or over the cells lining the secretory ducts.

accumulation is brought about by secretion in one part of the gland followed by reabsorption in another. Moreover, nothing as complicated as this could occur in other tissues, such as the mucous glands of the soft palate, in which iodide is also concentrated. However, as I have pointed out, there is reason to think that the mechanism for concentrating iodide in these tissues is similar to that in the salivary glands.

The simplest hypothesis is that the cells lining the secretory ducts transport iodide from the plasma to the lumen by a mechanism that is also operative in the cells of all those tissues in which iodide accumulation occurs. Fletcher *et al.* (1956), in their study of the metabolism of salivary glands *in vitro*, found that as the concentration of iodide in the medium was increased, there was a fall in the tissue/medium (T/M) iodide concentration ratio. Since the relationship between the T/M ratio and the iodide concentration in the medium conformed to Langmuir's adsorption equation, it was suggested that reversible adsorption of iodide to a substance in the salivary glands might be an essential step in the concentrating mechanism. Attempts to demonstrate, by means of dialysis experiments, the existence of a substance with a high affinity for iodide in homogenates of rat thyroid (Doniach and Logothetopoulos, 1955) and hamster submaxillary gland (Myant, unpublished) have not been successful. This does not disprove the existence of an adsorbing substance, since its stability might depend on the maintenance of an intracellular environment. However, an adsorbing substance in the cells of the salivary gland would not, by itself, account for the transport of iodide from the plasma, across the cell, and into the saliva. It would be hard to distinguish, simply from the relationship between iodide concentration and the concentration ratio achieved, a mechanism depending on adsorption from other mechanisms that include a step with a limited capacity for iodide. If, for example, the cell membrane was impermeable to iodide but permeable to some reversible carrier–iodide complex, the concentration ratio established across the membrane might be limited by the amount of carrier available, or by the rate at which it could return through the membrane for more iodide ions. A simple model for moving iodide across a boundary impermeable to iodide consists of a U tube containing a solution of iodide in each arm, separated by carbon tetrachloride in the bend of the tube. If a mild oxidizing agent is placed in the left arm and a reducing agent in the right arm, iodide will be oxidized to free iodine in the left arm and will then diffuse through the carbon tetrachloride to the right arm, where it will be reduced to iodide Provided the oxidizing and reducing agents are insoluble in carbon tetrachloride, the concentration of iodide on one side of the U tube will become higher than that on the other side. FIGURE 3 illustrates an experiment in which ferric chloride was the oxidizing agent and sodium arsenite the reducing agent. In this model, the concentration ratio finally established depends on the amounts of oxidizing and reducing agents added and the amount of iodide initially present in the U tube. When the amount of oxidizer in the left arm is nearly sufficient to oxidize all the iodide initially present, the relationship between the concentration of iodide at the beginning, and the concentration ratio finally established, deviates only slightly from the Langmuir equation. Demonstration that this relationship

holds in a biological system is clearly not proof that the mechanism depends on adsorption of iodide.

If iodide transport required the formation of a carrier–iodide complex and an intact cell membrane capable of maintaining a concentration gradient, the fact that homogenates do not concentrate iodide *in vitro* would be readily explained. However, there is no experimental support for such a mechanism. As I mentioned earlier, chromatographic analysis of salivary glands has shown

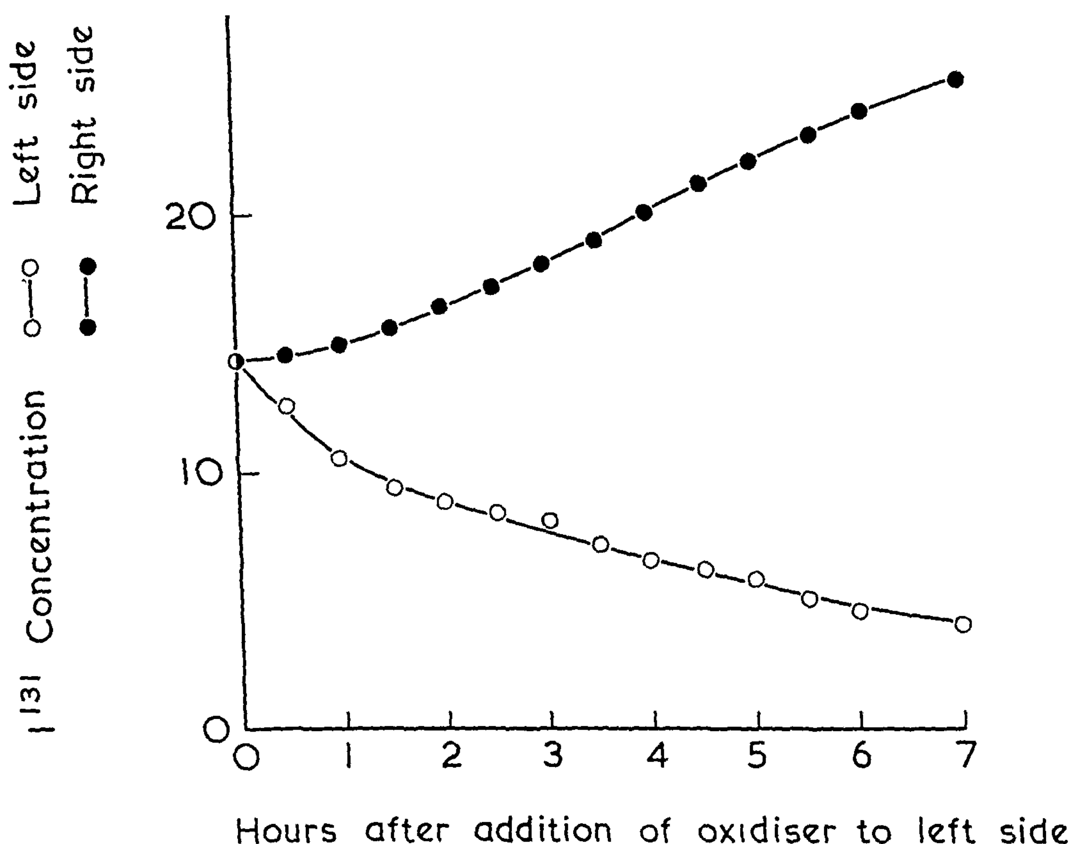


FIGURE 3. Transport of iodide from the left to the right arm of a U tube across carbon tetrachloride in the bend of the tube. At the beginning of the experiment, each arm contained 1 ml of 0 1 *M* radioactive KI in 0 1 N H_2SO_4 At zero time, 2 ml. of 0 1 *M* ferric-chloride was added to the left arm and 2 ml of 0 1 *M* sodium arsenite was added to the right arm The radioactivity was then measured in serial samples taken from each arm.

only iodide and two other iodinated compounds that are not concerned in iodide transport. If a carrier is concerned in iodide transport, the complex with iodide either must be very unstable, or must be present at a very low concentration.

In conclusion, the salivary glands of most species, along with several other tissues, including the thyroid gland, possess a powerful mechanism for concentrating iodide. The accumulation of iodide is inhibited by several anions which appear to act by competition with iodide. Thyrotrophic hormone, known to stimulate the iodide-concentrating mechanism in the thyroid, does not affect iodide accumulation in the salivary glands. In the salivary glands,

the mechanism is confined to the secretory ducts. Iodide is probably transported from the plasma, through the cells lining the secretory ducts, and into the saliva. Active transport of iodide may require either the presence of a substance inside the cell capable of adsorbing iodide, or the formation of a carrier–iodide complex to which the cell wall is permeable, or both may be necessary.

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ENDOCRINE INFLUENCES UPON THE SALIVARY GLANDS*

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Evidence is accumulating gradually that the salivary glands, at least in the experimental animal, are associated very intimately with various endocrine organs and that a functional disturbance of one of the latter may induce significant morphologic and physiological alterations in the salivary glands. Much of the early work concerned with the hormonal influences of the salivary glands was that of the Japanese, who published numerous papers speaking particularly of "the internal secretion of the salivary glands" (for example, Ogata, 1934; Takizawa *et al.*, 1938, 1939; Hukusima, 1940). The collective studies of the Japanese investigators are not entirely convincing in affirming that the salivary glands possess an endocrine function in themselves, although some of their work is quite suggestive.

Katagiri and Higashijo (1940) reported that ligation of rat parotid gland ducts and extirpation of the submaxillary glands resulted in atrophy of the testes and hypertrophy of the uterus and adrenal cortex. The salivary glands themselves have not been transplanted successfully from their usual location to another site in the body. Ota (1937) has reported that when the salivary glands of dogs were transplanted into bone marrow, the gland elements disappeared, but the ducts of the parotid gland persisted for some time.

An extensive report has been published by Ito (1954) on biochemical studies of a "purified salivary gland hormone" called parotin.

The French workers have made some of the most significant contributions in attempting to relate the salivary glands and endocrine organs. In the well-known papers by Lacassagne (1940 *a*, *b*, *c*), it was shown that there were histological differences between the male and female mouse submaxillary glands and that, if male sex hormone were administered to a female mouse, its submaxillary gland assumed the characteristics of that of the male. Lacassagne suggested that these organs could be used as a test of activity for androgens and estrogens, and speculated that, since a variation in submaxillary gland structure between sexes existed, a corresponding difference in the quality of the saliva excreted also might exist.

Subsequently, Raynaud and Rebeyrotte (1949 *a*, *b*) did report a difference in the amylase activity of mouse saliva between males and females. Junquiera and his co-workers (1948), however, were unable to find differences in amylase activity of male and female mouse salivary gland tissue extracts, although differences in the protease activity were noted. Buillard and Delsuc (1941) also have reported atrophic changes in the salivary glands following castration. Raynaud (1950) has shown that the injection of testosterone into the submaxillary glands of mice resulted in a marked hypertrophy of the tubules and interpreted this as evidence of a direct action of this adrogen on the gland.

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This sexual dimorphism of mouse submaxillary gland is a well-recognized phenomenon. Recently, Atkinson and his co-workers (1959) reported that the larger size of the glands in intact male and androgen-treated mice is due primarily to differential growth of the terminal secretory ducts. Furthermore, they found that the decrease in the weight of the glands after gonadectomy is due to different mechanisms in the male and female.

Of even greater interest, however, have been those changes occurring in the salivary glands that appear related to the pituitary and thyroid glands. Lacassagne and Chamorro (1940) reported extreme atrophy of the submaxillary gland following hypophysectomy and stated that the administration of androgenic hormone induced repair. In addition, it was shown by Arvy and Gabe (1950) that thyroidectomy induced a similar atrophy in the submaxillary gland, as did the administration of thiourea (Arvy *et al.*, 1950). The administration of thyroxine to a thyroidectomized animal produced a reversal of the atrophic changes. Reversal of such changes also followed thyroxine therapy in hypophysectomized animals (Gabe, 1950).

The studies of Eartly and Leblond (1954), however, did not confirm the latter finding. They reported that the administration of thyroxine alone to a hypophysectomized animal did not restore the submaxillary gland to its normal state, although thyroxine would restore the gland in thyroidectomized animals As a result, Eartly and Leblond suggested that the granular tubules of the submaxillary gland might be under the direct control of a hypophyseal factor. Interestingly, Grad and Leblond (1949) had reported previously that the simultaneous administration of thyroxine and testosterone was more effective in restoring the gland to its normal state in thyroidectomized animals than either hormone alone. This is of significance in the light of other papers in the literature that indicate a pronounced effect of testosterone on the thyroid gland.

Sreebny (1953) has studied the trypsinlike activity of the rat submaxillary gland in young animals, as well as in hypophysectomized animals. This enzyme exhibits trypsinlike activity, since it has characteristics of many of the properties of trypsin However, it is active in the presence of trypsin inhibitors. Sreebny reported that the trypsinlike activity of the rat submaxillary gland increases with the age of the animal and that the male usually exhibits a higher activity than the female. Since the limits of variation in activity were greater in the adult female than in the adult male, this suggested to Sreebny that this may be related to the estrous cycle. Sreebny also reported that the submaxillary gland protease activity in hypophysectomized animals is extremely low and corresponds to the atrophy known to occur in the granular tubules. On this basis he has suggested that the site of this enzyme is the granular tubule.

Studies in this general area, actually the experimental dental caries-endocrine organ-salivary gland axis, were begun in our laboratories more than eight years ago Originally, we were interested in studying particularly the relationship of the endocrine system to experimental dental caries in the rat, this study being prompted by the observations that the male hamster and male rat consistently develop more caries than the female of the respective species.

This is not related entirely to body size and food intake since, in the hamster, the female is the larger animal; in the rat, the male is larger. It also has been reported by Hodge (1943), by Keyes (1948), and by Shaw (1950) that castration reduces to some degree the incidence of experimental caries in both the rat and hamster.

Our initial studies (1952, 1953, 1954) were designed to repeat this original work, but were extended to include the administration of various hormones such as testosterone, methylandrostenediol, estradiol, and diethylstilbestrol (Muhler and Shafer, 1955). During the routine microscopic examination of tissues from these animals, it was noted that the salivary glands were markedly different from those of control animals; this immediately suggested that the alterations in caries incidence induced by the various endocrine procedures possibly could be mediated through the salivary glands.

A systematic investigation was begun in order to determine whether these observations could be related to the other endocrine organs as well. In retrospect and in the analysis of data collected in more than 7000 animals used in these studies, we now are convinced that the effects on caries and the salivary glands induced by hypo- or hyperfunctional states of the gonads are probably secondary to alterations induced by these procedures in the pituitary-thyroid axis.

Studies on the salivary glands of animals with altered function of the thyroid gland have shown marked alterations in their structure (Shafer and Muhler, 1956). Hypothyroidism, brought about by the administration of propylthiouracil, induces a marked atrophy of the so-called granular tubules. Before proceeding further, it would be appropriate to describe the particular structure in which these dramatic changes occur. The granular tubule appears to lie between the acinus and the intralobular excretory duct (FIGURE 1*a*). Normally, these tubules are small and few in number, and contain only occasional granules in the weanling animal. As the animal matures, the granular tubules increase in number and size, and become filled with these peculiar granules. The composition and function of these granules is not known, although Sreebny has suggested that they actually contain the proteolytic enzyme previously described. They are discharged into the excretory tubules apparently in the same form as they appear within the acinus It seems most reasonable that the presence or absence of these granules in the saliva could affect markedly the composition or consistency of the saliva and that this, in turn, could affect the caries incidence.

Hypothyroidism, then, produced by propylthiouracil in a weanling animal or an adult animal induces either lack of development or atrophy, respectively, of these tubules. They have a small diameter and are almost completely void of granules (FIGURE 2*a*). The administration of I^{131} in sufficient concentration to render the thyroid gland nonfunctional induces a similar atrophy of tubules (Muhler *et al*, 1956). The administration of either desiccated thyroid or purified thyroxin produces the opposite effect: an increase in the size of these tubules and an appearance of containing even greater numbers of granules (FIGURE 2*b*) Interestingly, the simultaneous administration of thyroid and testosterone produces a still greater enlargement of the tubules than

either thyroid or testosterone alone. This confirms the observations of Grad and Leblond (1949) that both testicular and thyroid hormones are necessary for the maintenance of the rat submaxillary glands.

Fawcett and Kirkwood (1954) have proposed that one of the important functions of the salivary glands is to control the level of thyroxine in the blood by deiodinating thyroxine and recycling the iodide ion to the thyroid gland via the saliva and gastrointestinal tract. Their work suggested that the level of thyroxine in the blood is controlled not only by its rate of synthesis of the thyroid hormone but also by the rate of degradation in the salivary glands.

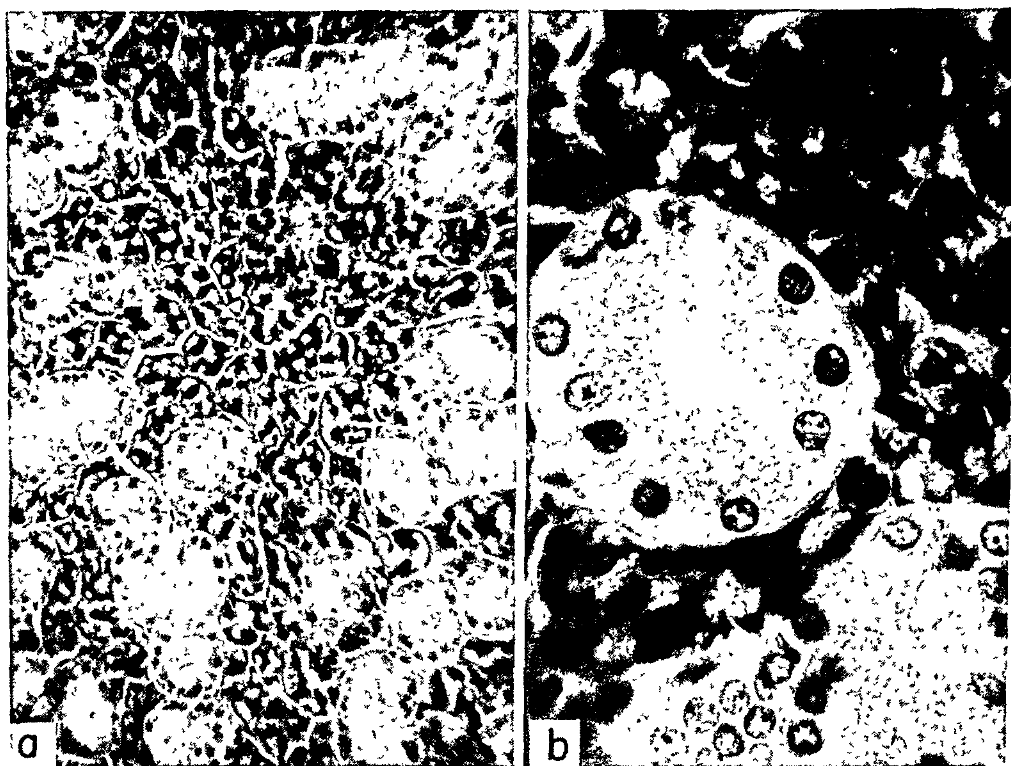


FIGURE 1*a* and *b*. Submaxillary gland, adult albino rat The size and distribution of the granular tubules are shown

Therefore, they postulated, the salivary glands function as reverse thyroid organs and have an important role in the extrathyroidal metabolism of organic iodide in the body. A subsequent report by Albright and his co-workers (1954) of hypertrophy of the salivary glands following the administration of triiodothyronine for myxedema in the human may be of some significance in the confirmation of this hypothesis. Ruegamer (1955), however, has disputed the evidence of Fawcett and Kirkwood and has shown that the salivary glands play no active role in the metabolism of thyroid analogues. He found that the amount of iodide cleared into the saliva appeared to be only a function of the circulating plasma level of iodide ion and was not influenced by thyroid hormone. Neither was he able to find a diiodotyrosine deiodinase system in the salivary glands of the dog. Previously Reiss and his co-workers

(1949 to 1950), in studies of radioactive iodine uptake and excretion in humans, have shown that the saliva of some patients contained up to 600 times more I^{131} per 100 mg. wet weight than the blood and stated "it appears that this concentrating activity of the salivary glands has some relation to the state of the thyroid function." This has been confirmed in hamsters by Cohen and his co-workers (1955), who found the concentration ratio of I^{131} in tissue and plasma to be for submaxillary gland, 5.3; parotid gland, 1.7; muscle, 0.4; and saliva, 28.0. However, these workers were not able to determine whether the high concentration of iodine in saliva was due to a direct secretion from duct

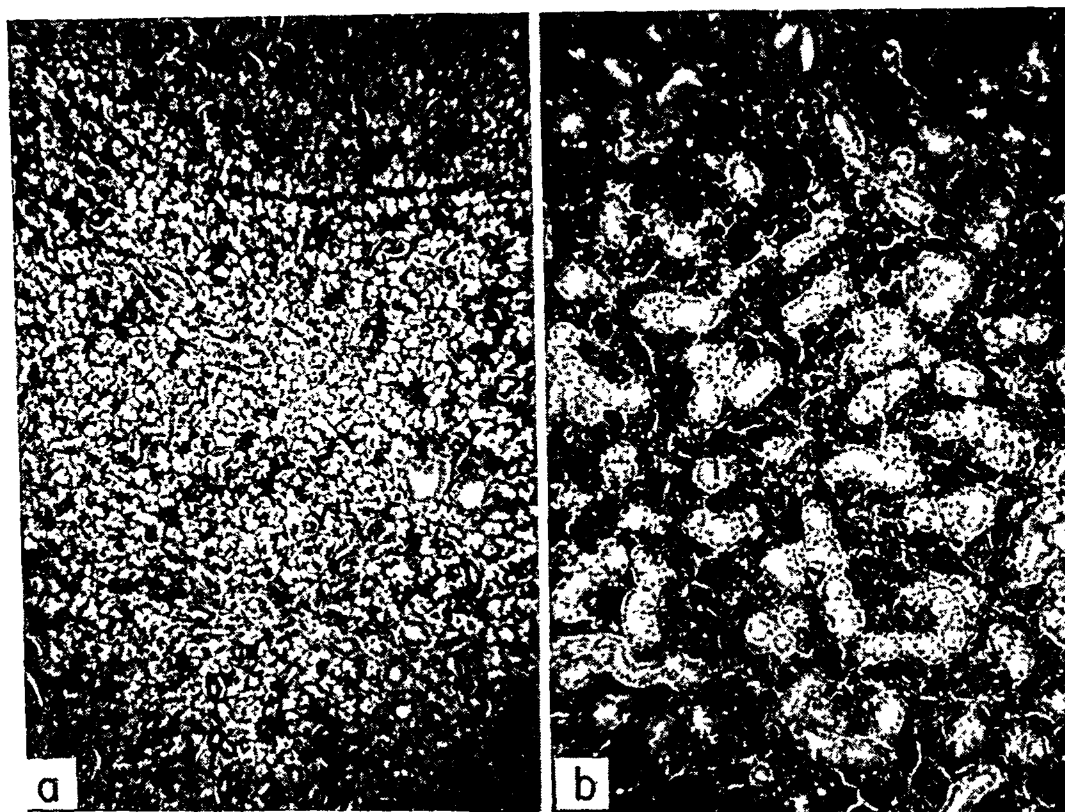


FIGURE 2 Submaxillary gland, adult albino rat Animal receiving propylthiouracil (*a*) exhibits decrease in size and number of granular tubules, while animal receiving thyroxin (*b*) shows numerous large granular tubules.

cells or to a selective reabsorption of water and other solutes from the saliva as it passes down the duct.

Wase and Feng (1956) have presented convincing evidence that sialoadenectomy reduces thyroid activity. Using P^{32} as an index of thyrotropic hormone (TSH) titer, they found that the reduced uptake of P^{32} by the thyroid glands occurred so rapidly following removal of the salivary glands that a direct relationship must exist between the two organs. Inasmuch as the inhibition of activity produced was proportional to the amount of salivary gland tissue removed, it appeared that if a single substance was responsible for the effect, it must be uniformly distributed or produced throughout all of the glands. An interesting observation of these authors was that the typical

reduced rate of growth that occurs in young sialoadenectomized animals could be reversed by the administration of bovine thyrotropic hormone.

Taurog and his co-workers (1959) have found that I^{131}, concentrated by the mouse submaxillary gland after its administration, remains in the form of inorganic iodide. This iodide-concentrating capacity of submaxillary gland tissue was not impaired by hypophysectomy nor by administration of TSH to either normal or hypophysectomized mice. It was concluded, therefore, that the submaxillary iodide pump, unlike the thyroid iodide pump, is not affected directly by the presence or absence of TSH.

Of equal interest but possibly of somewhat less importance in attempting to define the endocrine-salivary gland relationship have been the studies on hypophysectomized animals. We noted (1955), as other workers have, that hypophysectomy induces extreme atrophy of the granular tubules, the granules themselves almost completely disappearing (FIGURE 3*a* and *b*). This study corroborated the work of Sreebny to the effect that the proteolytic enzyme activity of these glands from hypophysectomized animals was diminished markedly In addition, we have reported that the amylolytic activity of salivary gland extracts was decreased as compared to controls, although the normal variation was quite wide (TABLE 1). These studies confirm the belief of Sreebny that the site of the protease activity is the granular tubule.

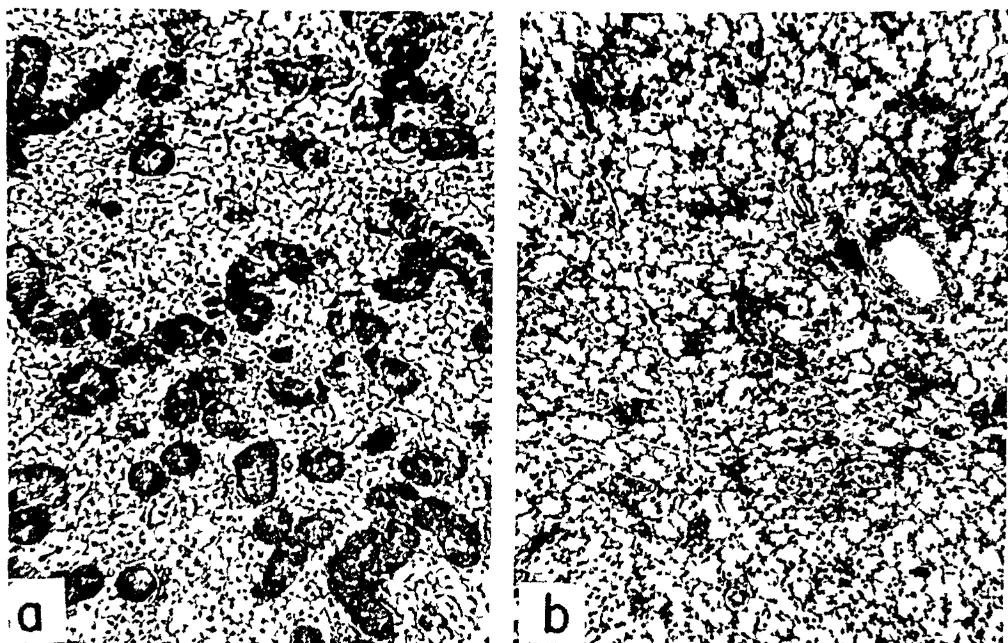


FIGURE 3 Submaxillary gland, adult albino rat. Control animal (*a*) and hypophysectomized animal (*b*).

Since the changes occurring after hypophysectomy were especially marked, it was only logical to attempt to find what particular factor or factors of the pituitary were responsible for the salivary gland changes and to attempt to prevent or reverse these changes by the administration of various hormones.

Studies on several groups of hypophysectomized animals have compared the histological appearance of the submaxillary glands with the proteolytic activity under the influence of a variety of hormones (Shafer *et al.*, 1956; Bixler *et al.*, 1959). Specifically, the ability of insulin, cortisone, estradiol, progesterone, purified pituitary growth hormone, thyroxine, testosterone, and thyroxine-testosterone in combination were evaluated for their ability to prevent loss of proteolytic activity following hypophysectomy (TABLE 2). The results of these studies indicate that insulin has no effect whatsoever on the salivary glands. Estradiol and progesterone have very minimal effects. Cortisone has a moderate effect, as does purified growth hormone. It is interesting to note that the latter substance was administered at a level that allowed growth comparable to that of control unoperated animals, yet the salivary

TABLE 1*

RELATIVE PROTEOLYTIC AND SALIVARY AMYLASE ACTIVITY OF THE SUBMAXILLARY SALIVARY GLANDS FROM CONTROL AND HYPOPHYSECTOMIZED RATS

Group	No of animals	Sex	Av final wt of animals (gm.)	Av. wt of submaxillary and sublingual gland (1 gland) (mg)	Proteolytic activity		Amylolytic activity	
					Relative activity†	Activity per gm ‡	Activity§ (mg /ml)	Range
Control	22	M	330	258	18 7	12 8	6 2	0 35–16 5
	23	F	194	182	15.7	19.2	12.5	3 82–21 8
Hypophysectomy	19	M	113	74	1 3	6 5	3 2	2 36–4 63
	20	F	100	78	1 7	5 6	1 8	0.70–3 7

* Reproduced by permission from *Journal of Dental Research* (Shafer and Muhler, 1955)

† Expressed as the relationship between the total gland activity and the activity of the tyrosine standard

‡ Expressed as the relationship between gland activity per gram of tissue and the activity of the tyrosine standard

§ Expressed as milligrams of reducing sugar liberated from starch by each milliliter of tissue extract.

glands were far smaller in size than those of the controls. This suggests that the effect on the granular tubules is not purely a somatic one or one of growth alone. Thyroxine and testosterone each partially prevent the regressive changes occurring after hypophysectomy. Of greatest interest, however, is the fact that, of all the materials tested, only the combination of testosterone and thyroxine completely prevented the regressive changes

Numerous studies on another salivary gland enzyme, arginase, have been conducted in an attempt to understand more about salivary gland function. Kochakian *et al.* (1955) have reported previously that arginase activity in mouse salivary glands was decreased by many of the cortical steroids, but was not influenced by thyroxine. Our own studies (1959) have shown an inverse relationship between protease and arginase activity in rat submaxillary glands under the influence of varying endocrine dysfunctions. For example, hypophysectomy increased arginase, but decreased protease activity. Thyroxine administration decreased arginase activity, but increased protease activity.

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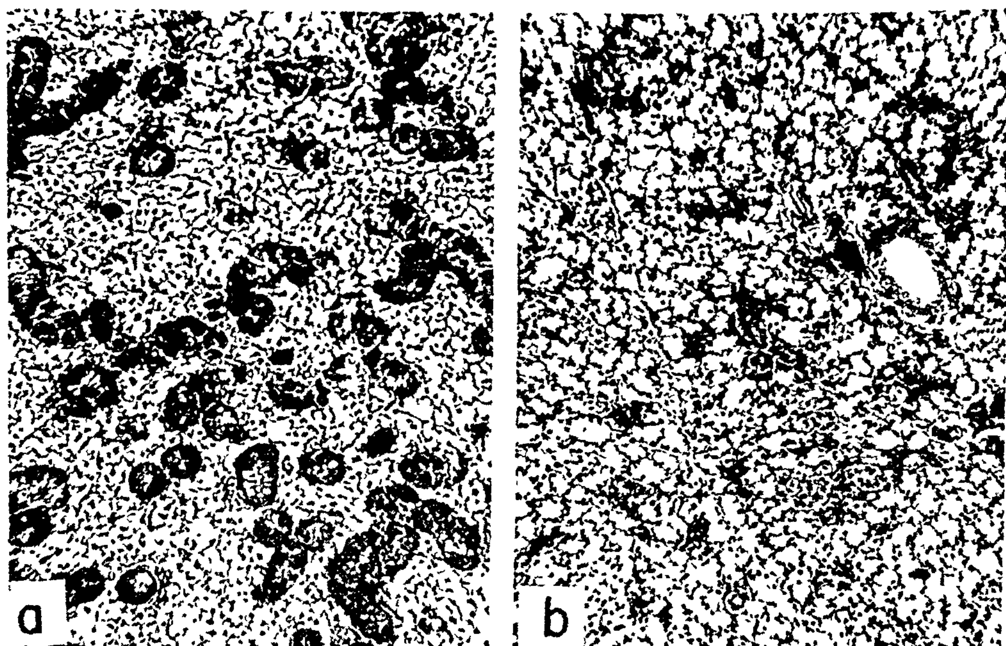


FIGURE 3. Submaxillary gland, adult albino rat. Control animal (*a*) and hypophysectomized animal (*b*)

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ministration to hypophysectomized animals did not restore RNA concentration to normal, whereas thyroxine and testosterone in combination did. Growth hormone partially restored RNA activity in the submaxillary gland of hypophysectomized rats. In the same studies, it was shown that a progressive loss of thyroid function produced by administration of graded doses of I^{131} resulted in progressive loss of RNA in the submaxillary and sublingual glands. Thyroidectomy did not appear markedly to affect the parotid gland. Related studies with graded doses of I^{131} showed a graded increase in dental caries concomitant with increasing doses of I^{131}. This paralleled a progressive atrophy of the submaxillary glands, evident chiefly in the granular tubules.

One of the more important aspects of this entire problem has been the attempt to correlate the known morphologic and histological changes occurring in the salivary glands subjected to various endocrine dysfunctions with functional alterations. This may help explain changes in dental caries experience occurring concomitantly with the endocrine disturbances.

With one notable exception, that being a radical decrease in salivary flow such

TABLE 4*

SALIVARY FLOW, VISCOSITY, AND *p*H IN RATS AS A FUNCTION OF AGE

Group	No of animals	Average body weight (gm.)	Average salivary flow (ml /hr)	Average viscosity (sec.)	Average *p*H
Weanling (26 days old)	10	76	1.0 ± 0.9†	75.2 ± 3.0†	8.6 ± 0.3†
Young adult (126 days old)	9	241	2.8 ± 1.5	49.9 ± 7.7	8.3 ± 0.3
Old adult (1 year old)	6	462	3.8 ± 0.7	48.9 ± 7.7	8.0 ± 0.2

* Reproduced by permission from *Journal of Dental Research* (Shafer *et al.*, 1958).
† Standard deviation.

as occurs in salivary gland extirpation or congenital aplasia, there have been no consistent alterations in salivary gland function demonstrated to be related to alterations in human or animal caries experience. Occasional workers have suggested that the viscosity of the saliva is related to caries experience. This has been denied by as many others, however. The findings with regard to salivary *p*H have been diverse and inconclusive. The content of the saliva with respect to such materials as various cations and anions, organic substances and enzymes, especially amylase, has not been related consistently to dental caries. Some of the inconsistencies may be due to the difficulty of studying such phenomena in the human subsisting, as he does, under different environmental conditions and eating different diets. This serves only to emphasize the need for studies of salivary function in small animals under controlled experimental conditions.

Preliminary investigations were begun to study the simplest salivary gland functions, total salivary flow, and viscosity in rats at varying ages. Data from this preliminary study (TABLE 4) indicate that salivary flow increases remarkably as the animal ages, while the viscosity appears to diminish significantly at least between weaning and young adulthood (Shafer *et al.*, 1958). The increase in salivary flow might be expected, since normal flow is apparently de-

These findings have suggested that the site of the arginase is probably the acinar cells, since these are diminished per unit volume when the granular tubules increase in size and number. The significance of the enzyme arginase is not known, however.

TABLE 2*

SUBMAXILLARY GLAND TUBULAR SIZE AND PROTEOLYTIC ACTIVITY OF HYPOPHYSECTOMIZED RATS RECEIVING VARIOUS HORMONES

	Final weight of animals (gm.)	Average total weight gain or loss (gm.)†	Weight of gland (mg.)	Average diameter of tubules (μ)	Proteolytic activity	
					Relative activity‡	Activity of tissue§
Control	355	+39	268.8	45.3 ± 2.9**	13.68	10.41 ± 0.68**
Cont. hypo	238	−44	103.6	28.1 ± 1.1	2.13	4.23 ± 0.83
Insulin	250	−24	127.3	31.0 ± 0.8	2.80	4.55 ± 0.78
Cortisone	180	−87	132.1	37.6 ± 1.0	5.31	8.25 ± 0.50
Estradiol	222	−59	89.9	28.3 ± 0.6	2.56	5.82 ± 0.49
Progesterone	244	−24	120.4	31.9 ± 1.3	3.63	6.26 ± 0.69
GH	296	+37	161.5	38.1 ± 2.4	6.93	8.76 ± 0.83
Thyroxine	211	−69	150.2	39.1 ± 2.2	7.16	9.89 ± 1.25
Testosterone	251	−32	168.8	36.5 ± 1.4	7.44	9.04 ± 0.42
Testosterone-thyroxine	210	−65	201.5	44.8 ± 1.2	11.00	11.22 ± 0.78

* Reproduced from Shafer *et al.*, 1956. Courtesy of Charles C Thomas, publisher.
† Calculated as the difference in weight of animals at the beginning of the experimental study 7 days posthypophysectomy and at the termination of the study.
‡ Expressed as the ratio between the color produced by the total gland activity and the color of the tyrosine standard.
§ Expressed as the ratio between color produced by the gland activity per gram of tissue and the color of the tyrosine standard.
** Standard deviation.

TABLE 3*

EFFECT OF HYPOPHYSECTOMY AND HORMONE REPLACEMENT THERAPY UPON RNA CONTENT OF THE MAJOR SALIVARY GLANDS

	Submaxillary gland	Sublingual gland	Parotid gland
Hypo + testosterone + thyroxine	BND† 4‡	BN† 4‡	BND† 3‡
Hypo + cortisone	BN 1	BN 1	BN 1
Hypo + testosterone + thyroxine	BN 3	BN 2	BN 2
Hypophysectomized	BN 0	B 0	BN 1
Control unoperated	BN 2	B 2	BN 2

* Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* (Bixler *et al.*, 1957).
† Denotes location of RNA. Key: B, basal portion of cell, N, nucleolar, D, diffusely spread in cytoplasm.
‡ Denotes estimated amount of RNA as judged by intensity of staining reaction. Key: 0, essentially none present, 4, very heavy concentration of RNA.

Other studies in our laboratories have been concerned with the ribonucleic acid localization and concentration in the salivary glands. It has been found (Bixler *et al.*, 1957) that only the acinar portion of the gland exhibits such activity, none being found in the duct system. Following hypophysectomy, the RNA is reduced markedly in the acini of all glands (TABLE 3). Cortisone ad-

mized with radioactive iodine and maintained for a period of three months without further treatment. The data indicated that the salivary flow of the thyroidectomized animals after this three-month period was extremely low and that the salivary viscosity was increased remarkably as compared to that of control animals (TABLE 6). Following these determinations, the administration of thyroxine was begun to each animal in the thyroidectomized group, and salivary flow and viscosity were periodically determined. By the ninth day, the salivary flow was increased so that it approximated the level of the control group, while the viscosity decreased to a level identical with that of the controls.

Thus, it appears that thyroxine replacement therapy in radiothyroidectomized animals will return the salivary flow and viscosity to limits comparable to those of control animals within a relatively short period. While no conclusions can be drawn, it is interesting to note the parallelism between the effect of replacement therapy in returning caries scores to those of the control level, as reported on several occasions by this laboratory, and in returning salivary flow and viscosity to control levels. While salivary flow and viscosity here appear to exhibit an inverse relationship, there is no direct evidence that these are necessarily associated. Their relationship remains to be determined.

In the short time available, it has been impossible to review more than a small part of the existing literature on the hormonal influences of the salivary glands. Furthermore, only a small part of the studies carried out in our laboratories has been cited, no attempt being made to correlate these data with the vast amount of caries data collected, which has been the basic problem of interest to us.

There is little doubt in our own minds or in the minds of others close to this problem that the salivary glands, their morphology, physiology and pathology, are related very closely to the endocrine system, although the nature of this relationship appears to be a very complex one. Definition of this relationship may do much in the future to solve some of the unexplained facets of the problem of dental caries and other oral diseases of man.

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pendent, at least to some extent, on the total circulating blood volume and the size of the salivary glands. The explanation for the decrease in viscosity is not immediately apparent, although it could indicate a change in the proportion of the total saliva with respect to its serous and mucous components

With these studies as a foundation, the effect of thyroid interference on salivary gland function was investigated next because of the known effect of this

TABLE 5*
EFFECT OF THYROXINE AND THIOURACIL ON RAT SALIVARY FLOW

Group	No of animals	Average body wt (gm)	Salivary flow (ml /hr)
Control	4	360	2 9 ± 0 6†
Thyroxine	9	356	4 3 ± 1 2
Thiouracil	5	273	1 6 ± 0 4

* Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* (Shafer *et al* , 1958)
† Standard deviation

TABLE 6*
EFFECT OF THYROXINE ON SALIVARY FLOW AND VISCOSITY IN RADIOTHYROIDECTOMIZED RATS

Group	No of animals	Average body wt (gm)	Salivary flow (ml /hr)	Salivary viscosity (sec)
		0 days		
Control	8	267	1 6 ± 0 5†	54 ± 12
I^{131}	10	126	0 1 ± 0 1	1100 ± 26
		3 days		
Control‡	8	275	1 1 ± 1 1	46 ± 7
I^{131} + thy ‡	10	123	0 1 ± 0 1	86 ± 14
		5 days		
Control	8	257	2 6 ± 1 0	58 ± 14
I^{131} + thy.	10	126	0 4 ± 0 3	73 ± 20
		9 days		
Control	7	260	1 8 ± 0 7	49 ± 7
I^{131} + thy	8	130	1 2 ± 0 8	49 ± 4

* Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* (Shafer *et al* , 1958)
† Standard deviation
‡ Thyroxine

procedure on histological alteration of the rat salivary glands as previously described. In one study (Shafer *et al.*, 1958), animals received propylthiouracil or thyroxine over a ten-week period, after which salivary flow was determined. It was found that the thyroxine group secreted considerably more saliva than the control animals, while the propylthiouracil group showed a remarkable diminution in flow (TABLE 5). These data are consistent with the general view that decreased salivary flow is accompanied by an increase in the incidence of dental caries.

In the same series of studies, a group of weanling animals was thyroidecto-

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Japan (for example, the endemic Aso-volcanic disease), arthritis deformans, spongylitis deformans, and alveolar pyorrhea (chronic destructive periodontal disease). Parotin was confirmed to be effective in compensatory therapy for the diseases mentioned above.

Bioassay of Parotin and Parotinlike Substances

Parotin and parotinlike substances are biologically active in (1) lowering the serum calcium level in rabbits and other animals (calcium activity), (2) decreasing and later increasing the number of circulating leukocytes in rabbits (leukocyte activity), and (3) promoting the calcification of incisor dentine in rabbits and rats (dentine-calcification activity). Calcium activity was used for quan-

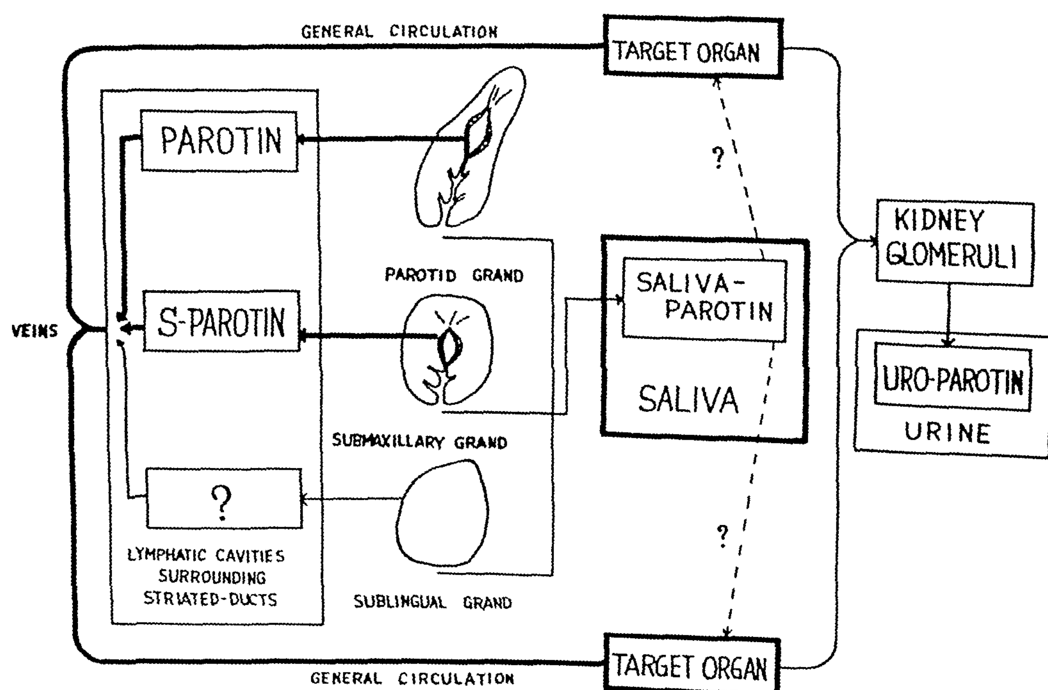


FIGURE 1 Schematic diagram of the interrelationship of parotin and parotinlike substances.

titative estimation, and the others for qualitative identification, in bioassays of parotin and parotinlike substances in the course of studying their isolation and physicochemical or chemical properties.

Calcium Activity Assay Method[3]

Early in the course of studies on the isolation of parotin from the bovine parotid gland, parotin was assayed histologically in measurements of the promotion of calcification of teeth and alveolar bones in rats. Thereafter, my associates and I discovered[3] that parotin lowered the calcium level in rabbit serum remarkably and that the mode of such lowering was quite specific for parotin; furthermore, the calcium activity was nearly proportional to that estimated from the histological test mentioned above Therefore, the study of the intrinsic nature of the relation between the calcium reduction and the calcification effect was put aside, and the calcium activity was used in a standard assay

PAROTIN: A SALIVARY GLAND HORMONE

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The endocrine function of the salivary glands has been under study in Japan for more than thirty years, ever since T. Ogata of the University of Tokyo started research on the experimental pathology of the salivary glands. During this time, I and my co-workers isolated an active principle, parotin, in pure crystalline form from the bovine parotid gland; we surmised that it was the salivary gland hormone, as Ogata and his associates claimed.

Later this active substance was investigated closely by many workers of the consolidated research group;* not only was light thrown on its chemical nature, but it was established that this substance is able to compensate experimental animals and human beings for deficiencies in the parotid gland.

Furthermore, my associates and I isolated active principles in the form of homogeneous proteins having biological activities similar to those of parotin from bovine, porcine, and equine submaxillary glands (S-parotin), from human saliva (saliva parotin), and also from human urine (uroparotin) (FIGURE 1).

This paper deals with the chemistry and biochemistry of parotin and the parotinlike substances described above.

The Endocrine Function of the Salivary Glands[1,2]

Among the three major salivary glands, (namely, the parotid, submaxillary, and sublingual), the parotid, with the cooperation of the submaxillary gland, plays the principal role in endocrine functioning. The sublingual gland is said not to participate in this function.

The salivary gland hormone (hereafter referred to as parotin) is formed in the parotid gland. The epithelial cells of the striated tubules (*Streifenstuck*) of the salivary ducts absorb parotin together with other saliva contents being excreted into the oral cavity, and increte them into the general circulation via the lymph cavity surrounding them.

In experimental animals, parotin deficiency symptoms (asialadenism) followed extirpation of both the parotid and submaxillary glands, and a hyperfunctional state (hyperasialadenism) resulted from ectomy of the submaxillary gland or the administration of parotin. The intimate interrelationship of the functions of the parotid gland and those of the various other endocrine organs was also confirmed.

Parotin generally acts on the mesenchymal tissues, especially the hard and connective tissues, to promote their development and growth (FIGURE 27). It has also a protein-anabolic function.

The primary diseases resulting from parotin deficiency in human beings are shown to include chondrodystrophia fetalis, Kaschin-Beck's disease (endemic in Manchuria, China), the endemic diseases occurring in the volcanic districts in

* Organized with the support of a grant-in-aid for Scientific Research from the Ministry of Education, Tokyo, Japan.

cluded that this increase in the number of leukocytes was not related to the adrenal body. Later S. Hara, using rabbits, confirmed these findings. Much remains to be studied thoroughly before it can be determined whether this effect of parotin on the circulating leukocytes is characteristic of parotin. However, judging from Tasaka's report concerning the effect of parotin on bone marrow temperature, this reaction seems to be specific to parotin.

In anticipation of possible application of this reaction to the bioassay of parotin, we examined this leukocyte activity statistically, using the 4 × 4 Latin square method on 4 rabbits. One-milliliter doses of 0.6 per cent saline solution containing 5.4, 9.0, 15.0, and 25.0 mg. of purified parotin were injected intravenously into the animals. The blood was taken just before the injection and also 1, 2, 3, 4, 5, 6, 8, 10, and 12 hours after the injection. Once used, the rabbits were not used again until 5 days later, to eliminate the effect of blood loss. The number of leukocytes was counted on the Thoma blood-counting plate with

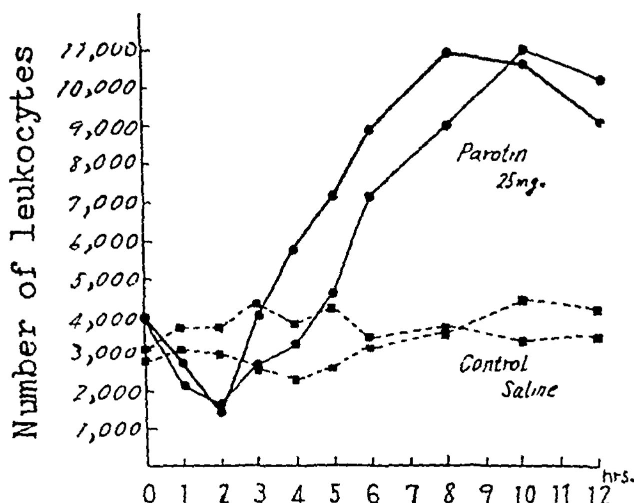


FIGURE 3. Effect of parotin injection on the number of circulating leukocytes in rabbits (Reproduced with permission from *Endocrinoligia Japonica* [3])

Turk's solution on fresh blood and, for accuracy, at least 3 samples were prepared for each measurement.

As shown in FIGURE 3, intravenous injection of 25 mg. of parotin caused a transitory decrease followed by an increase in the number of leukocytes. The maximum decrease appeared 2 hours after the injection and the maximum increase at around 8 to 10 hours after the injection (the percentages of decrease or increase 1, 2, 6, 8, 10, and 12 hours after the injection were all significant). A statistical analysis was performed on the variances due to differences in treatment, days, and rabbits for the parotin dose range of 5.4 to 25.0 mg., and the linearity was examined. It was found from this analysis that the treatment did not affect significantly the initial increase in leukocytes that is considered to be due specifically to parotin. However, the treatment caused significant variances in the transitory decrease occurring from 2 to 3 hours after the injection. The variances due to rabbits and days were also significant. A linearity test showed significance of the linear but not the higher-order term. In

for parotin, the histological test of calcification being inconvenient for the chemical study of parotin.

The following assay method was used. Male rabbits of more than 2 kg. body weight were kept in individual cages on a fixed diet for at least 1 or 2 months. Three to 5 rabbits, in which the maximum variation in serum calcium level did not exceed 5 per cent (as determined from 2 or 3 blood drawings a day), were taken from this group. After fasting for 24 hours, the rabbits were injected intravenously with amounts of the sample solution in proportion to their body weights. During the 8 hours following injection, blood was drawn at 3 definite intervals and the lowest calcium value was measured. The maximum per cent decrease against the calcium level before injection was determined. The minimum effective dose per kilogram of rabbit body weight was taken to be that

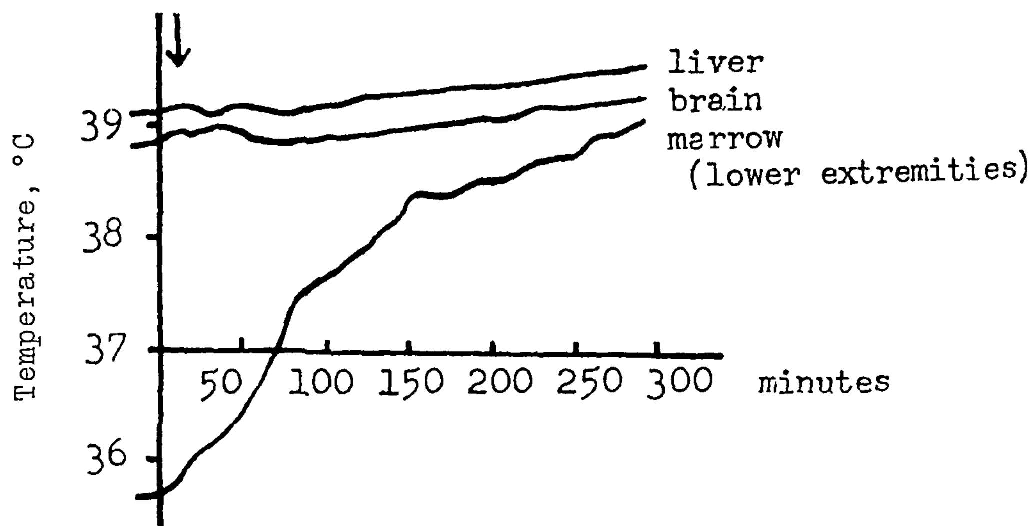


FIGURE 2 Elevation of bone marrow temperature after intravenous injection of parotin (5 mg /kg body weight) in rabbits Room temperature 18 to 19° C, humidity 60 to 70 per cent (Reproduced with permission from *Nipponrinsho* [99])

which caused, on the average, 13 to 15 per cent calcium decrease in 3 to 5 rabbits.

Leukocyte Activity Assay Method[3]

S. Tasaka found that the injection of parotin into the aural vein of rabbits caused a selective rise in bone marrow temperature (FIGURE 2), he concluded that parotin must act directly on the hematopoietic function of the marrow. On examining the bone marrow picture and hemogram, he found that these showed changes in a definite direction, due to parotin injection.

On the other hand, Takaoka[4] noticed that upon injection in rats of 10 mg. of parotin/kg of body weight, the number of circulating leukocytes decreased very rapidly within the first 2 to 3 hours after the injection, but increased markedly from 5 to 8 hours and up to 24 hours after the injection It was found that this first decrease occurred in intact rats and not in adrenalectomized rats, while the subsequent increase occurred even in adrenalectomized rats. Thus he con-

jections in rats of 1 mg./kg. body weight over 4 successive days promoted calcium deposition during administration, followed by a return to the normal state; on the other hand, 30 mg./kg. body weight administered in the same way caused a notable inhibition during the 4 days of injection, followed by a slightly promotive effect.

The following is an example of the assay method used.[7] Four separate injections of 0.2 ml. of 1 per cent lead acetate solution per kg. body weight were injected at intervals of 2 to 3 days, each injection being given at the same time of day. Ten minutes after the second injection of lead acetate solution, 0.1 mg. of saliva parotin A (a parotinlike substance to be described later) per kg. body weight was injected. After the animals were sacrificed, the incisors of the mandibles were removed, fixed in formaldehyde solution, stripped of calcium by immersion in 0.2 *N* hydrochloric acid saturated with hydrogen sulfide, and frozen. Sections of the incisors were then stained with hematoxylin.



FIGURE 5 Parotin crystal ×150; precipitate at 12 1 per cent ammonium sulfate, *p*H 6 8 (Reproduced with permission from *Journal of the Pharmaceutical Society of Japan* [8])

When 0.01 mg. of saliva parotin A per kg. body weight was injected into a rabbit, deeply stained stripes were produced immediately after the injection, clearly revealing accelerated calcification (FIGURE 4*a*). However, when a dose 10 times larger—0.1 mg of saliva parotin A per kg. body weight—was injected, the inhibition of calcification was observed as unstained stripes followed by deeply stained stripes (FIGURE 4*b*). The irregularity of the stained stripes indicates the incompleteness of the calcification. Therefore, it is clear that an excessive dose of saliva parotin A results in an inhibition of calcification. The same is true in the case of parotin, as already reported by Sato.[6]

Parotin, the Parotid Gland Hormone

In 1944 a biologically active substance of protein nature that promoted the calcification of the hard tissues in rats and decreased the serum calcium level in rabbits was isolated by my associates and myself in a crude form from the bovine parotid gland and named parotin Subsequently, we endeavored to purify it and finally, in 1949, we succeeded in obtaining parotin in a homogeneous crystalline form (FIGURE 5).

addition, when the values were adjusted for the body weight and the initial value, the value 2 hours after injection was found to be significant.

These statistical results on the change in the number of leukocytes suggest that this reaction may not be suitable for quantitative assay. Therefore, the reaction is used only for qualitative identification of parotin and parotinlike substances, and is carried on simultaneously with calcium activity determination.

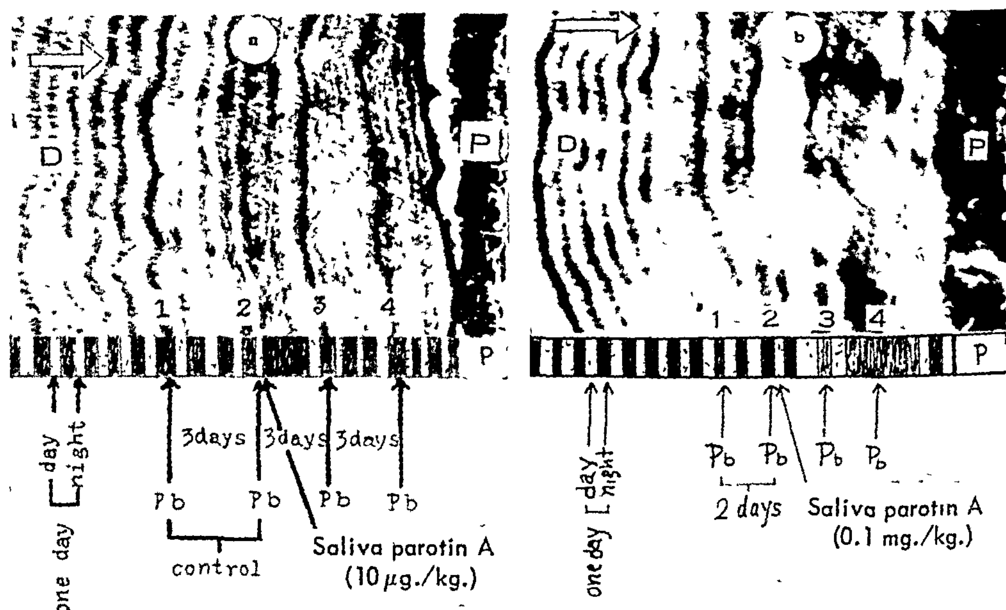


FIGURE 4 Dentine-calcification activity of saliva parotin A in rabbits transverse section of rabbit incisor dentine stained with hematoxylin D, normal dentine, arrow, direction of formation of dentine, P, pulpa Numbers show the time interval between lead sulfide lines, 3 days in (*a*) and 2 days in (*b*) In (*a*), a dose of 10 µg saliva parotin A per kg body weight was injected just after the second lead sulfide line; in (*b*), a dose of 100 µg saliva parotin A per kg. body weight was injected just after the second lead sulfide line

DENTINE-CALCIFICATION ACTIVITY ASSAY METHOD

The tendency of parotin to promote calcification of incisor dentine in rats or rabbits was demonstrated clearly with the Okada-Mimura lead acetate vital staining time-marking method [5]

Sato[6] determined that the minimum effective dose of parotin was 0 3 mg./kg. body weight in a single intravenous injection, and 3 mg /kg. body weight in a single subcutaneous injection. Larger amounts, such as 3 mg./kg body weight by intravenous injection and 5 mg./kg. body weight by subcutaneous injection, caused not only inhibition of calcification and deterioration of the dentine quality, but also a decrease in growth rate. Sato also found that the single subcutaneous injection was effective for a comparatively longer period. Employing the lead acetate vital staining method, Okada found that 0 5 mg of parotin/kg body weight (given in 3 injections over 3 consecutive days) promoted the deposition of calcium in rat incisor dentine, but that daily subcutaneous in-

due to charge effects with protein molecules when measurements are made in a solvent of ionic strength less than 0.1. If this is the case with parotin, the value of 800,000 may be higher than the true molecular weight. Meanwhile, our recent experiments on the ultracentrifugal sedimentation and boundary spreading of parotin have suggested that its molecular weight may be considerably lower than that determined previously. For this reason, my associates and I[9] reinvestigated the molecular size and shape of parotin by measuring sedimentation constants, diffusion constants, and intrinsic viscosity at an ionic strength of 0.2 (TABLE 1). In the reinvestigation we found a molecular weight of parotin of 132,000, assuming a slightly hydrated prolate ellipsoid molecule with an axial

TABLE 1
SOME CONSTANTS OF HOMOGENEOUS PAROTIN RELATING TO SEDIMENTATION, DIFFUSION, AND VISCOSITY

Sedimentation constant $s_{20,w}$	3 81 × 10^{-13} (cm /sec)(dyne/gm.)
Diffusion constant $D_{20,w}$	2 84 × 10^{-7} cm ^{2}/sec.
Intrinsic viscosity [η]	0 428
Partial specific volume V	0 753
Frictional ratio calculated from s and D, f/f_0	2 21
Molecular weight calculated from s and D, $M_{s,D}$	132,000

TABLE 2
MOLECULAR SIZE AND AXIAL RATIOS OF PAROTIN CALCULATED FROM s AND D

Molecular shape	Hydration (gm water/gm protein)	Axial ratio (a/b or b/a)	$2a$* (Å)	$2b$† (Å)
Prolate ellipsoid ($a > b$)	0 0	25 7	592	23
	0 3	19.4	492	25
Oblate ellipsoid ($a < b$)	0 0	39 7	6	232
	0 3	27 9	7	206

* Length of the axis of rotation
† Length of the axis perpendicular to $2a$

ratio of 25 7. The size and axial ratios of parotin molecule estimated from the values in TABLE 1 are shown in TABLE 2.

The molecular weights, axial ratios, and frictional ratios (TABLE 3) were calculated by use of the value of volume fraction intrinsic viscosity, ν, that is, the quotient of intrinsic viscosity [η] by partial specific volume V. As seen in TABLE 3, the value of $M_{s,\nu}$ coincides with the value of $M_{D,\nu}$ only when the parotin molecule is assumed to be a prolate ellipsoid and to contain no water of hydration. In this case the molecular weight is 132,000, the frictional ratio is 2.2, and the length of the long ($2a$) and short ($2b$) axes are 592 and 23 Å, respectively. The parotin molecule has been assumed to contain little water of hydration, but in general a protein molecule is thought to contain normally about 0.3 gm. of water of hydration per gm. of protein in aqueous solution. Therefore, it is necessary to estimate the amount of water of hydration assuming that some experimental errors are included in the values of s, D, and [η] FIGURE 7 shows the relation-

Extraction and Isolation[8]

The crude parotin was obtained by extraction of the ground bovine parotid gland with weak basic aqueous solution (*p*H 8.0), followed by repeated precipitation of the effective principle at the isoelectric point (ca. *p*H 5.4).

Ammonium sulfate was added to the crude parotin solution up to a concentration of 7 per cent, and the precipitated inactive material was discarded. Ammonium sulfate was added to the supernatant until its concentration was 22.5 to 25 per cent. The precipitate of the effective component was separated by centrifugation and dialyzed. More ammonium sulfate was added to this dissolved precipitate, bringing the concentration up to 15.2 per cent, at which the effective component precipitated in a crystalline state. This precipitate was dialyzed again and more ammonium sulfate was added, up to a concentration of 12.1 per cent. The crystalline precipitate obtained was either dialyzed further and lyophilized or precipitated once again at the isoelectric point, dialyzed,

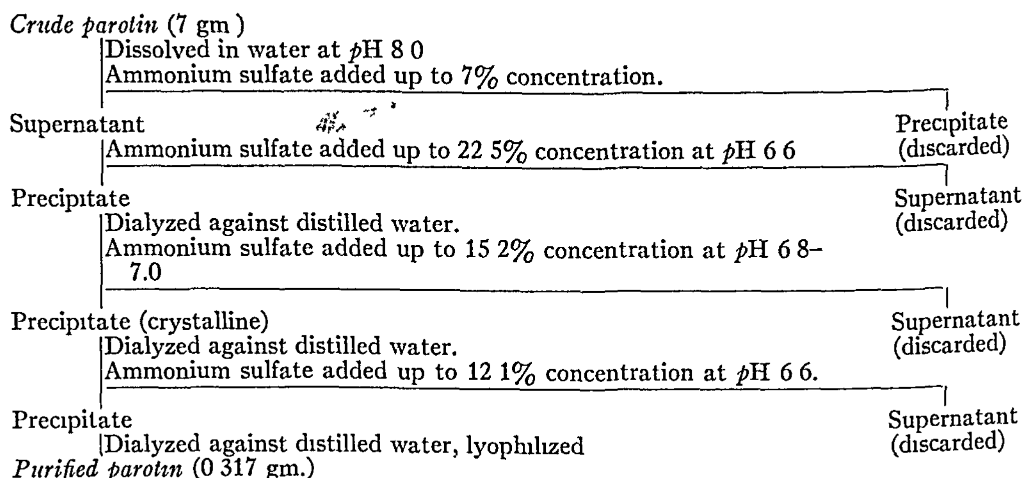


Figure 6 Flow sheet of the parotin purification process.

and lyophilized. The precipitate from the 15.2 per cent ammonium sulfate solution was found to be 92 per cent homogeneous by electrophoresis, and is now used for clinical therapy. After repeating the precipitation twice from the 15 2 per cent ammonium sulfate solution, we obtained an almost pure homogeneous crystalline precipitate by adjusting the solution to a concentration of 12 1 per cent ammonium sulfate. The yield of a 92 per cent pure preparation is 0 02 per cent of the weight of the parotid gland, less fats and adhering tissues (figure 6).

Physicochemical Properties of Parotin

The homogeneous parotin was found to be a protein of globulin nature having an isoelectric point of *p*H 5.7 (as measured with an antimony electrode).

Weight and Shape of the Parotin Molecule[9]

It was reported previously[3] that the molecular weight of parotin was estimated at about 800,000 from measurement of the diffusion constant and intrinsic viscosity in a phosphate buffer of ionic strength 0 083. It is well known, however, that the diffusion constant gives a high value of molecular weight,

parotin molecule is assumed to contain from 0.0 to 1.0 gm. hydrated water per gm.

M. Ogata estimated the molecular weight of parotin to be about 128,000, using a Zimm plot of light-scattering data, without assuming a particular shape for the molecule.

It remains to be determined, however, whether the previously obtained low value of diffusion constant is due only to the charge effect or to changes in the molecular size, such as those brought about by association of parotin molecules, as well. Although we have not observed the association of parotin molecules over a wide range of protein concentrations, it may occur under suitable conditions of *p*H, ionic strength, and other such factors. In order to clarify these problems, investigations are now being carried out concerning the molecular kinetics of parotin under various conditions.

Ultraviolet Absorption[3]

In acidic (*p*H 1.0), neutral (*p*H 7.0), or slightly alkaline (*p*H 8.0) solutions, parotin showed an ultraviolet absorption maximum at 277 ± 0 5 mμ, and in strongly basic (*p*H 11.0) solution the absorption maximum shifted to 287 mμ. This is characteristic of parotin and the extinction value ($E^{1\%}_{1\ \mathrm{cm.}}$) at that wave length increased as the purity of parotin increased. The concentration of parotin showed a relationship linear with extinction, and an absorption maximum at 277 mμ in the concentration range of 0.1 to 0 8 mg. per ml.

Polarography[3]

Characteristic polarograms were obtained for parotin in ammonium chloride and ammonium hydroxide buffer solution, in the presence of bivalent or trivalent cobalt ions, over a range of −0 8 to −2.0 volts. The polarogram consisted of 3 waves (FIGURE 38), the second of which was shown to be characteristic of parotin protein, with a half-wave potential of −1.35 volts, referred to the standard calomel electrode in the presence of cobaltous or cobaltic ions. The height of the second wave increased with the purity of the parotin and showed a relationship linear with parotin concentration in the range of 0.05 to 0.15 mg per ml of solution.

Chemical Properties

*Stability of Activities**

Solid parotin containing less than 5 per cent moisture can be stored for a long time (for example, 4 years) in a desiccator at room temperature without noticeable loss of activity, but when it contains more than 7 per cent moisture it loses its calcium activity very rapidly under the same conditions.

As shown in TABLE 4, parotin in aqueous solution of *p*H 8.0 maintained its calcium activity for 9 hours of incubation at 37° C and for 5 hours at 60° C. After 24 hours of incubation, however, even at 37° C. the activity was lost completely.

Parotin in aqueous solution (*p*H 8.0) retained leukocyte activity for 5 hours

* Ito and Shinoda, unpublished

ship between the amount of hydrated water and the axial ratio of molecule, the curves being drawn on the assumption that the values of s, D, and $[\eta]$ might include ±5, ±5, and ±10 per cent errors, respectively. The curved surface calculated from s and D overlaps that calculated from η in the range of $1/32$ to $1/16$ on the ordinate; this area indicates the possible interrelationship of the axial ratio and the amount of hydrated water. On the basis of this calculation, the

TABLE 3
AXIAL RATIO, FRICTIONAL RATIO, AND MOLECULAR WEIGHT OF PAROTIN CALCULATED FROM s AND ν OR D AND ν

Molecular shape	Hydration (gm water/ gm protein)	a/b or b/a	f/f_0	$M_{s,\nu}$	$M_{D,\nu}$
Prolate ellipsoid ($a > b$)	0 0	25.4	2.20	132,000	132,000
	0.3	20.6	2 29	137,000	122,000
Oblate ellipsoid ($a < b$)	0 0	82 6	2 79	188,000	65,000
	0.3	58 4	2.79	188,000	65,000

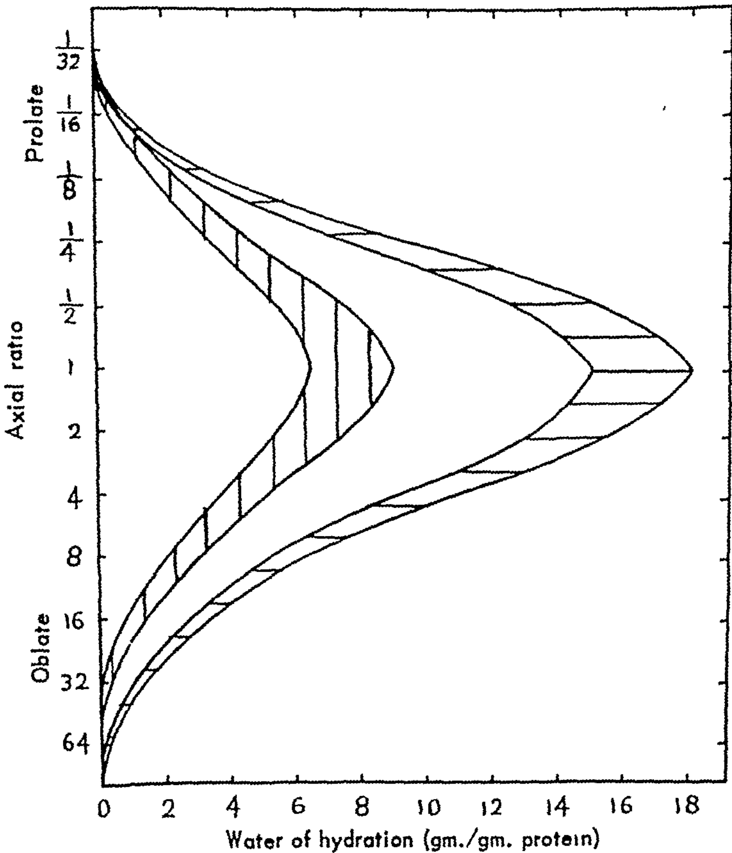
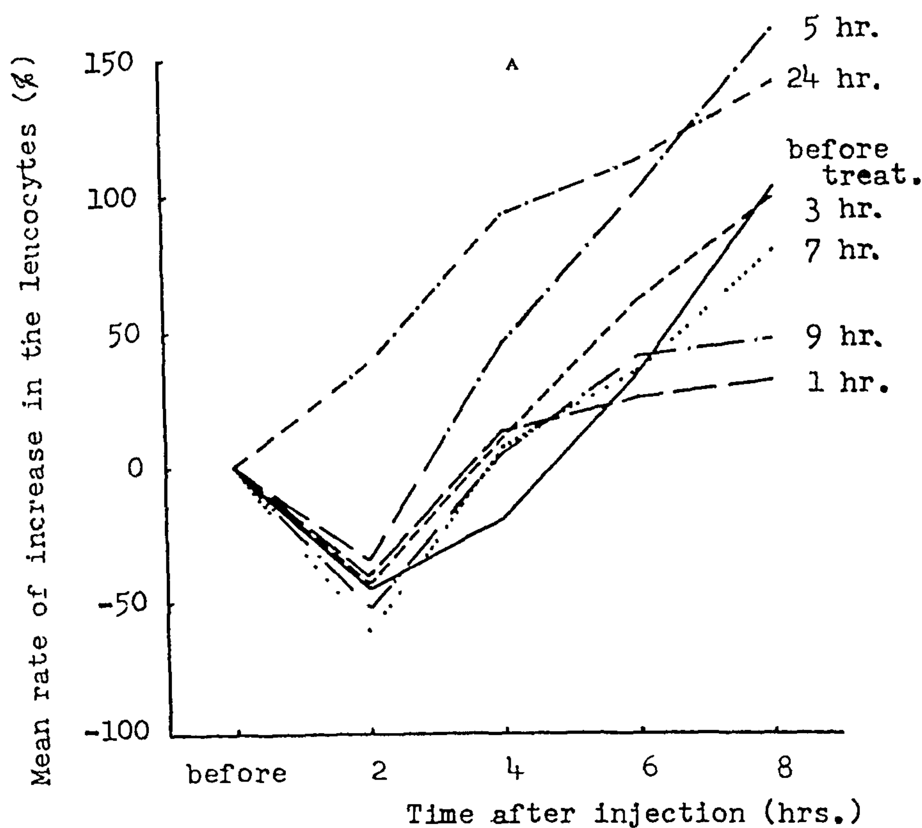


FIGURE 7. The relationship between amounts of water of hydration and the axial ratio of the molecule. Horizontally cross-hatched areas calculated from ν; vertically crosshatched areas, from s and D.



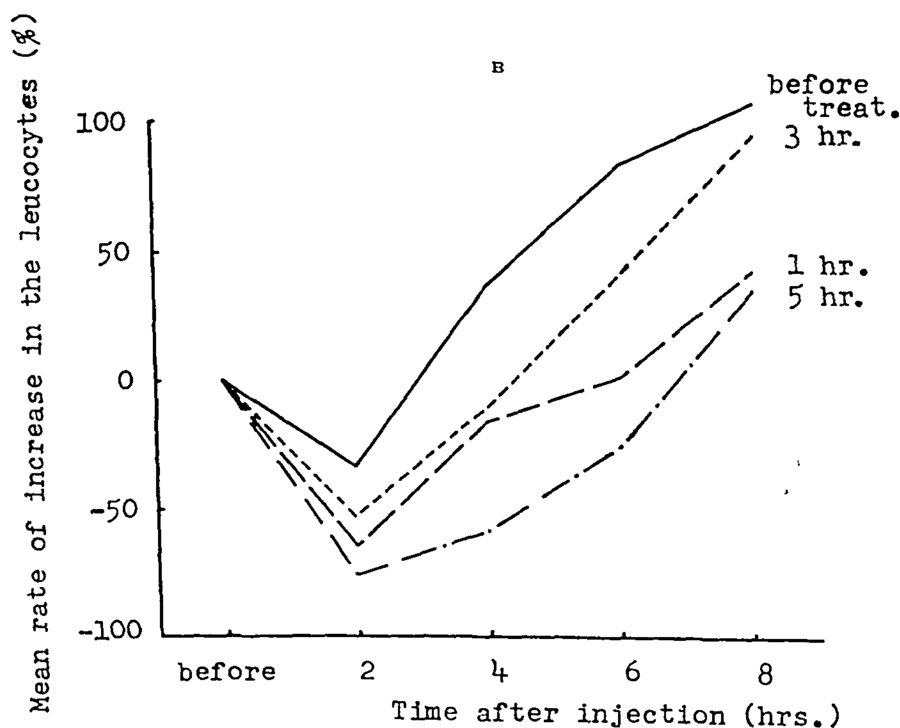


FIGURE 8 Stability of leukocyte activity of parotin in aqueous solution at pH 8.0 (A) Incubation at 37° C, 1 mg. of parotin per kg body weight injected intravenously into rabbits, (B) incubation at 60° C, dosage as in (A).

at 60° C , but after 24 hours of incubation at 37° C. the initial decrease in the number of leukocytes disappeared and only the succeeding increase remained (FIGURE 8)

Although the calcium activity of parotin is labile in strong acid solution (*p*H 1.4) and decreases with increasing length of treatment, it is remarkably stable in strong basic solution (*p*H 12.4), being retained perfectly even after 24 hours at 5° C. or after 5 hours at 37° C. (TABLE 5).

In contrast to the calcium activity, the leukocyte activity of parotin is stable in acid solution and very unstable in basic solution. Thus, as seen in FIGURES 9 and 10, in basic solution the initial leukocyte decrease lessened remarkably even after 3 hours of incubation at 5° C.; after 24 hours at the same temperature or after 3 hours at 37° C., not only the initial leukocyte decrease but also the succeeding increase disappeared.

TABLE 4
HEAT STABILITY OF CALCIUM ACTIVITY OF PAROTIN IN SLIGHTLY BASIC SOLUTION

Treatment			Calcium activity* Mean ± S E †
*p*H	Temperature (°C)	Hours	
8 0	37	0	13 78 ± 1 63
		1	10 94 ± 0 99
		3	12 27 ± 1 83
		5	11 46 ± 1 01
		7	12 02 ± 1 73
		9	13 89 ± 2 70
		24	2 06 ± 2 06
8 0	60	0	11 95 ± 0 61
		1	13 45 ± 0 89
		3	9 40 ± 2 38
		5	10 95 ± 2 14

* Dose 1 mg per kg body weight intravenous injection in rabbits
† Standard error.

As shown in FIGURE 11, the ultraviolet absorption maximum remained unchanged at 277 mμ after 24 hours of incubation at 37° C The extinction coefficient at that wave length remained constant in a solution of *p*H 7 0, but showed slight increases in solutions of *p*H 1 0 and *p*H 11.0 after incubation at 37° C. Possibly, a relationship exists between the decrease of the calcium or leukocyte activity and the increase in the extinction coefficient

Although the biological activities and ultraviolet absorption spectra of parotin were altered by treatment in strong acid or basic solution, no degradation or splitting of the molecule was observed with either paper partition chromatography or paper electrophoresis

Composition of Parotin[3]

Elementary analysis of the homogeneous preparation showed the following percentage composition· C, 50.84; H, 7 31, N, 14 53; P, 0.06; S, 0.77 The amount of ash was negligible. The nitrogen from the amino groups was 0.6

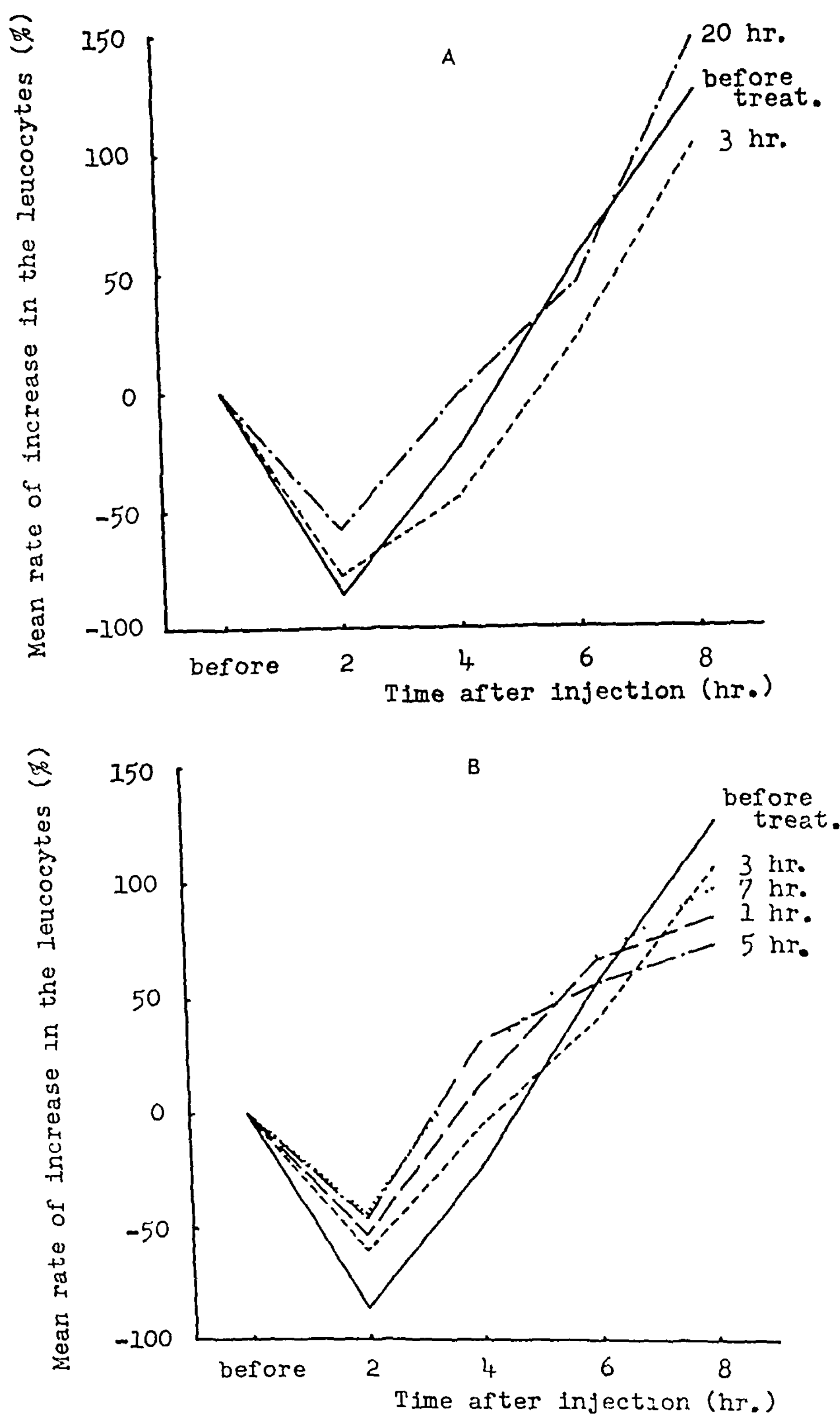


FIGURE 9 Stability of leukocyte activity of parotin in acidic solution (*p*H 1 4) (*A*) Incubation at 6° C, 1 mg of parotin per kg body weight injected intravenously in rabbits; (*B*) incubation at 37° C, dosage as in (*A*).

per cent and the percentages of certain amino acids were as follows: tyrosine, 3.64; tryptophan, 1.09; cystine (containing neither the —SH group nor cysteine), 1.13. Even in pure parotin, minute quantities of hexose, but no glucosamine, were present.

By paper partition chromatography, the hydrolyzate of 92 per cent pure parotin was shown to consist of the following 17 amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The N-terminal amino acids were identified as aspartic acid, glycine, and alanine. The ε-lysine was also found.

TABLE 5
STABILITY OF CALCIUM ACTIVITY OF PAROTIN IN ACIDIC AND BASIC SOLUTION

Treatment			Calcium activity* Mean ± S E †
*p*H	Temperature (°C)	Hours	
8 0	Control (untreated)		10 55 ± 0 76
1 4 (acidic)	6	3	9 20 ± 1 06
		20	7.72 ± 1 99
	37	1	7.02 ± 0 81
		3	6 70 ± 1 48
		5	4.30 ± 1 58
12.6 (basic)	5	3	15.27 ± 0 99
		24	11 75 ± 0 64
	37	3	11 29 ± 1 28
		5	10 93 ± 1 14

* Dose. 1 mg. per kg body weight intravenous injection in rabbits
† Standard error

Effects of Various Chemical Reagents on the Calcium Activity[3]

In studying the significance to the calcium activity of the free amino radical, the tyrosine phenyl radical, and the disulfide bond in the parotin molecule, these were eliminated or blocked by various reagents. For example, the amino group was blocked or eliminated with ketene, nitrous acid, or formaldehyde; the phenyl radical in tyrosine residues was substituted with iodine in neutral solution; and the disulfide bond was reduced with thioglycollic acid. These reagents either considerably diminished or destroyed the activity. From these findings it may be concluded that for parotin, as in the case of other proteohormones, no one radical is solely responsible for the activity: the free amino radical, the tyrosine phenyl radical, and the disulfide bond are all responsible. The effect of the denaturation of the parotin by urea and guanidine hydrochloride was examined. The activity disappeared upon addition of urea, but reappeared after urea was eliminated through dialysis and upon standing for some time. However, the activity was destroyed irreversibly upon addition of guanidine.

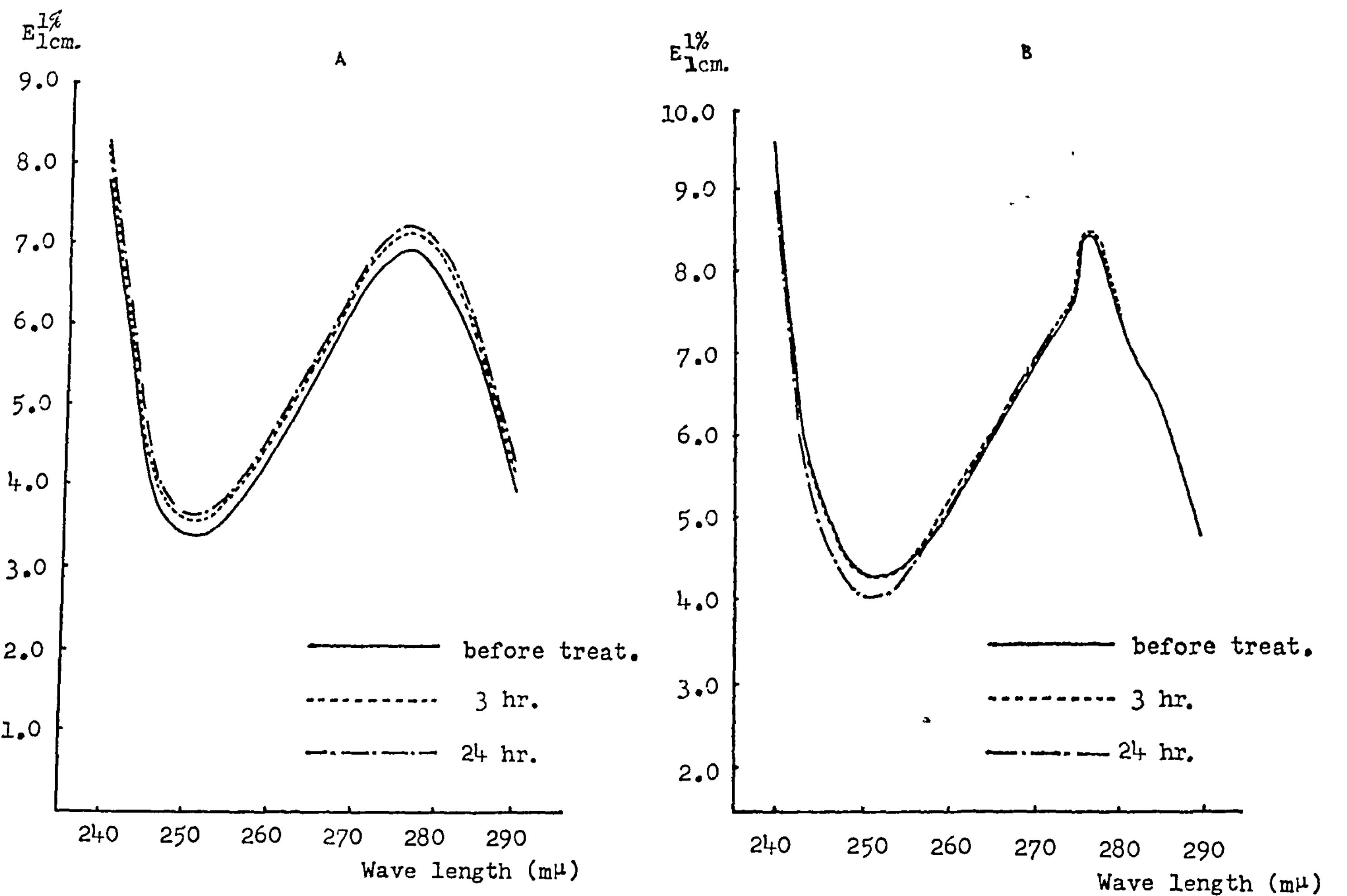
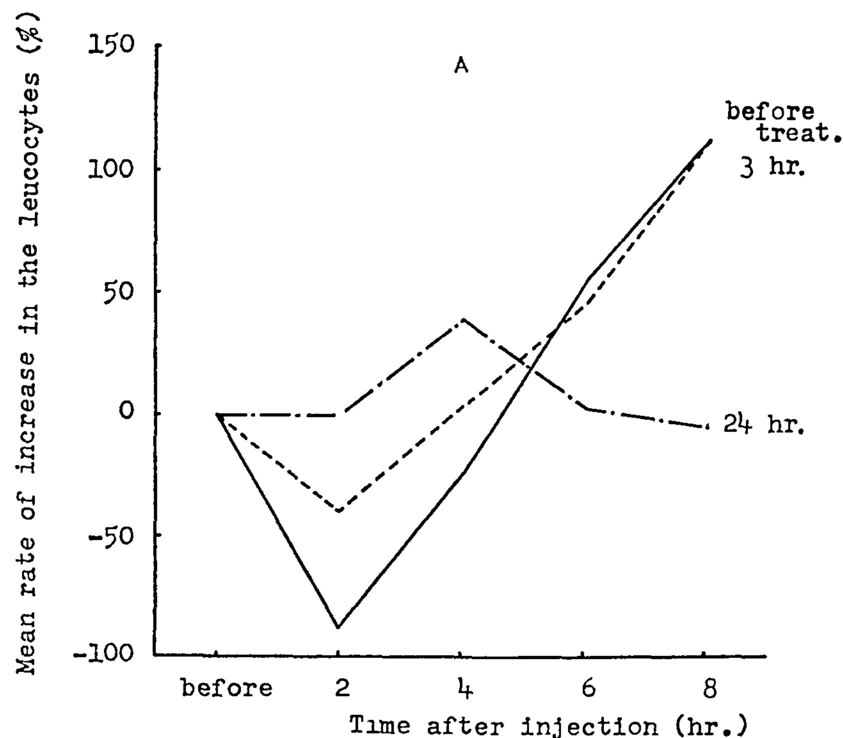


FIGURE 11. Ultraviolet absorption spectra after incubation at 37° C. in (*A*) acidic solution (*p*H 1.0) and (*B*) neutral solution (*p*H 7.0)



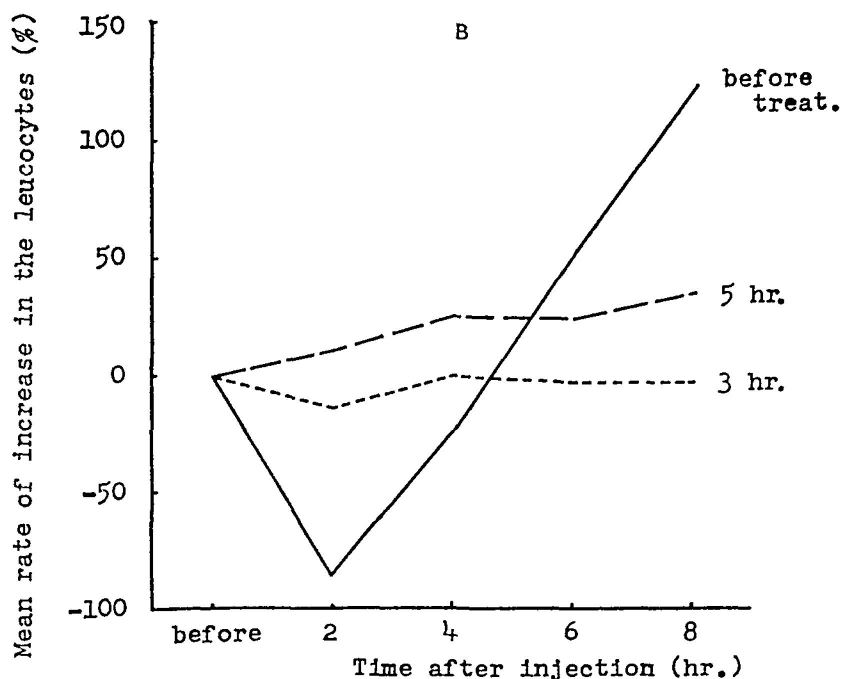


FIGURE 10 Stability of leukocyte activity of parotin in basic solution (*p*H 12 6). (*A*) Incubation at 5° C., 1 mg of parotin per kg. body weight injected intravenously in rabbits, (*B*) incubation at 37° C., dosage as in (*A*)

chloroacetic acid-soluble fraction (FIGURE 15b). The trypsin and intact parotin were not developed with this solvent.

Chymotrypsin. As shown in TABLE 7 and FIGURE 16, in the preliminary treatment of parotin with chymotrypsin in a solution of *p*H 8.0 at 37° C., neither calcium nor leukocyte activity was affected within the 7 hours of treatment.

The Distribution and Metabolism of Administered Parotin

Distribution of I^{131}-Labeled Parotin in Vivo[12,13]

Parotin and horse serum albumin (the latter prepared by the method of McMeekin[17]) were labeled with radioactive iodine by the method of Sonenberg,[16] with some modification. The biological activity of the iodinated parotin has been largely retained.

The labeled proteins were injected intravenously (0.5 mg. per rat) into male

TABLE 6*
INFLUENCE OF TREATMENT WITH TRYPSIN ON CALCIUM ACTIVITY OF PAROTIN

Incubation time† (hr)	Without trypsin (A)		With trypsin (B)		*t* Test§ (A:B)
	No of rabbits	Percentage of serum calcium-decreasing activity‡ Mean ± S E	No of rabbits	Percentage of serum calcium-decreasing activity‡ Mean ± S E.	
0	5	13.78 ± 1.22	—	—	—
1	5	10.94 ± 0.99	6	8 71 ± 0.81	not significant
3	5	12.29 ± 1.83	9	11.23 ± 0.87	not significant
5	4	11.46 ± 1 01	5	6 53 ± 0.98	significant
24	3	2.06 ± 2.06	6	1.49 ± 1 38	not significant

* Reproduced by permission from *Endocrinologia Japonica.*[10]
† Incubating temperature: 37° ± 0 5° C.
‡ Dose of parotin: 1 mg /kg. intravenous injection
§ Level of significance: 0 05.

rats weighing about 100 gm., under Nembutal anesthesia. The localization of radioactivity in the blood, seminal vesicle, prostate, adrenal, thyroid, hypophysis, testis, kidney, spleen, liver, thymus, pancreas, muscle, femur epiphysis, and femur diaphysis (marrow and cortex) was measured 5, 20, 60, and 180 min. after injection (FIGURES 17, 18, and 19). Exclusive of the thyroid, which accumulated the inorganic iodide freed from the labeled parotin, the largest amount of radioactivity was found in the liver, the next largest amount in the kidney, and the next largest in the spleen. These results are compared with those of Sonenberg *et al.*[16,18,19] on I^{131}-labeled ACTH, prolactin, growth hormone, and bovine serum albumin in FIGURE 20. The characteristically high concentration of radioactivity in the liver and spleen was found only in the case of the labeled parotin. The values for the kidney, however, were not necessarily characteristic of parotin.

Rapid increase of radioactivity in the thyroid was observed only after the administration of labeled parotin (FIGURE 21). In the case of the labeled horse

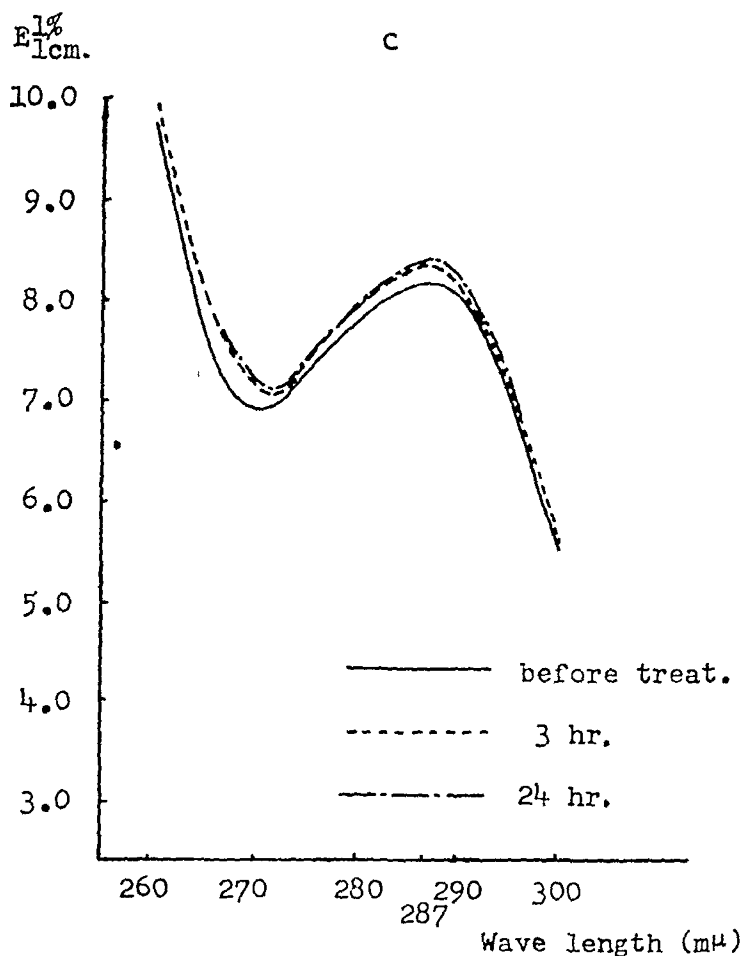


FIGURE 11. (C) Ultraviolet absorption spectrum after incubation at 37° C in basic solution (pH 11 0).

Behavior against Proteases[10,11]

Trypsin. As shown in TABLE 6, calcium activity of parotin was destroyed completely after 5 hours of incubation with trypsin at 37° C. As for the leukocyte activity, the initial decrease was destroyed completely and the successive increase was retained after incubation with trypsin (FIGURE 12).

As shown in FIGURE 13, the amount of free amino nitrogen in the tryptic digest of parotin increased rapidly during the first hour of incubation and continued to increase slowly for 24 hours of incubation. As in the case of free amino nitrogen, the optical densities at the absorption maximum (277 mμ) also increased very rapidly within 1 hour of incubation in the digested solution (curve A, FIGURE 14), in the supernatant of the digest precipitated at pH 5.4 (curve B, FIGURE 14), and in the supernatant of the digest precipitated at 5 per cent trichloroacetic acid (curve C, FIGURE 14). After 1 hour of incubation, densities increased at a considerably slower rate. On the paper partition chromatogram of the tryptic digest of parotin, two spots were recognized at R_f 0.08 and 0.13 after half an hour of incubation. The same spots were always found after 1, 2, 3, 4, 5, and 24 hours of incubation (FIGURE 15a) and also in the tri-

in liver and spleen was due to the modification (iodination) of the parotin molecule, the following experiments were carried out.[13] A group of 12 male rats was divided at random, as regards litters, into 3 groups for 3 different treatments, as shown in TABLE 8. The radioactivity localized in the liver, kidney, spleen, and blood was measured 5 min. after the injection (FIGURE 23). Group B rats, injected with the labeled parotin and parotin, had remarkably less radioactivity localized in the liver than had the control groups A and C (with and without

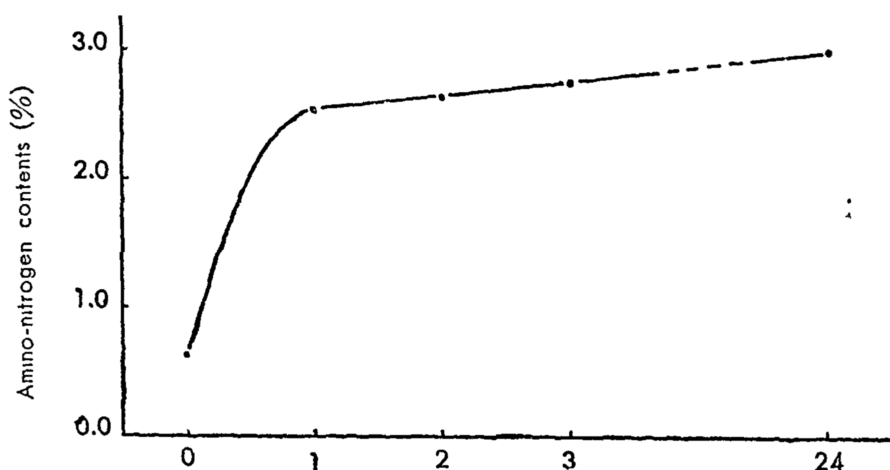


FIGURE 13. Change in amino nitrogen content after 1, 2, 3, and 24 hours of treatment with trypsin (Reproduced with permission from *Endocrinologia Japonica* [11])

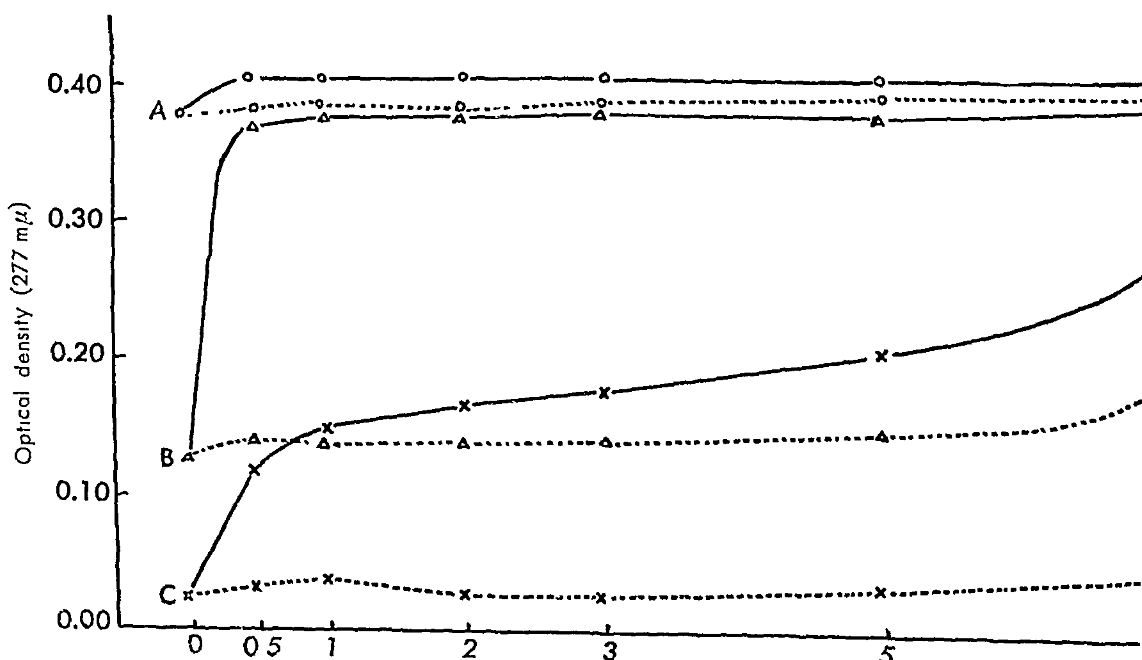
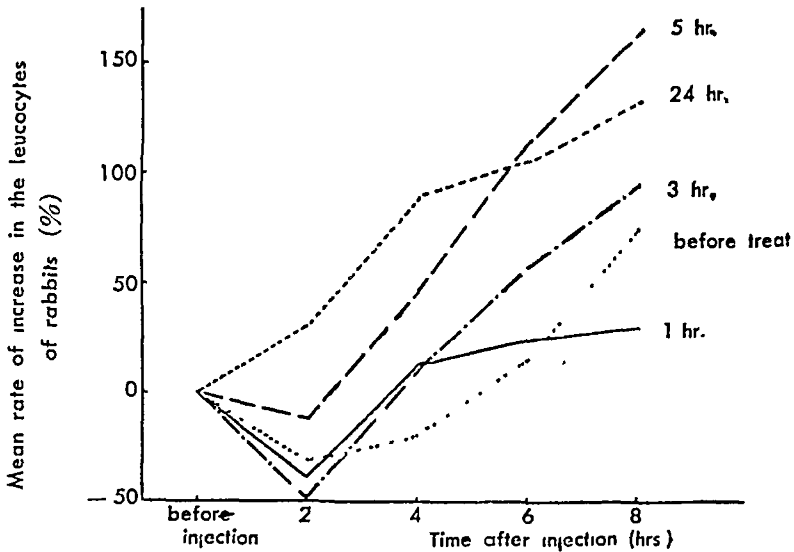


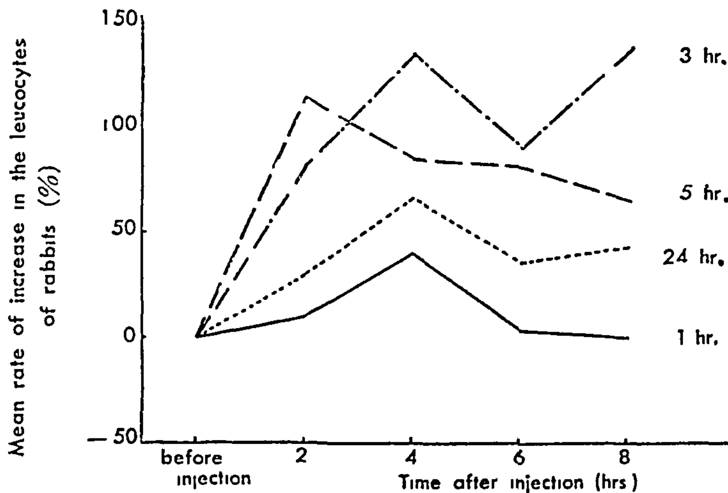
FIGURE 14 Optical densities at 277 mμ after 1, 2, 3, 5, and 24 hours of treatment with trypsin Curve A, acetic acid solution; curve B, supernatant solution of precipitation at pH 5 4; curve C, supernatant solution of precipitation with 5 per cent trichloroacetic acid (Reproduced with permission from *Endocrinologia Japonica* [11])

serum albumin, more than 99 per cent of the radioactivity in plasma was found to be associated with the trichloroacetic acid-precipitable fraction from 5 to 180 min. after administration, during which time a gradual degradation of the labeled parotin was observed (FIGURE 22).

To determine whether the characteristic accumulation of the labeled parotin



A



B

FIGURE 12 Leukocyte activity of parotin after treatment with trypsin (*A*) Control experiment, (*B*) treatment with trypsin (Reproduced with permission from *Endocrinologia Japonica* [10])

rotin as well as I^{131}-labeled parotin would localize characteristically in the liver and spleen. Similar experiments suggested that parotin and labeled parotin would be degraded *in vivo* in a similar manner.

Degradation of I^{131}-Labeled Parotin in Vivo[14]

The effect of ligation of several blood vessels on the degradation and distribution of the labeled parotin in rats was studied. The degradation of labeled parotin, measured as the percentage of total administered radioactivity found

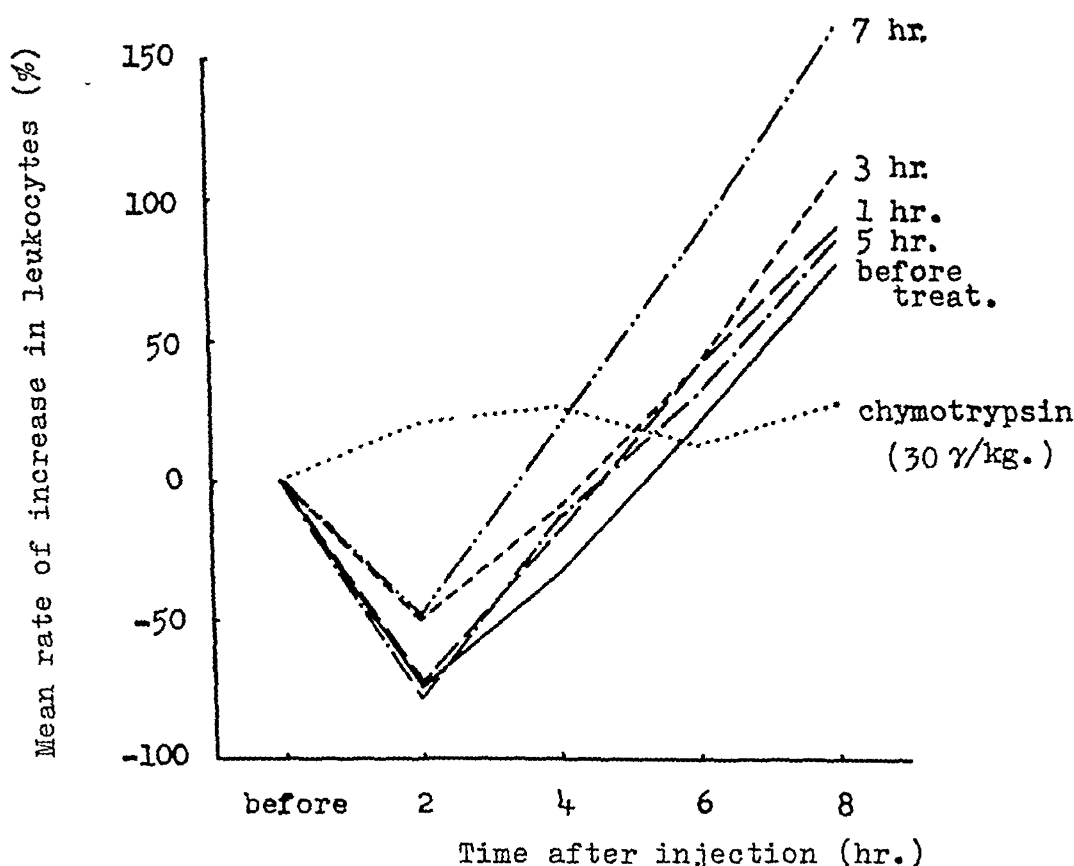


FIGURE 16 Leukocyte activity of parotin after treatment with chymotrypsin. One mg. of parotin per kg body weight was injected intravenously into rabbits.

in the nonprotein fraction of plasma, decreased noticeably following the ligation of the celiac artery or renal vessels. Degradation of labeled parotin was least in the group of rats whose portal vein and celiac, superior mesenteric, and inferior mesenteric arteries were ligated.

It was found that, following the ligation of the celiac artery, the liver still accumulated as much labeled parotin as did the livers of control rats. It was observed also that the rate of disappearance of radioactivity from the liver decreased greatly, as did the rate of degradation of the labeled parotin.

The degradation and distribution of the labeled parotin in the liver and the kidney were closely correlated. These organs were recognized as the main sites of the degradation.

bovine serum albumin). Differences between A and B, and also between B and C, were found to be highly significant ($p < 0.01$). No statistically significant difference was found between A and C. Significantly less radioactivity was

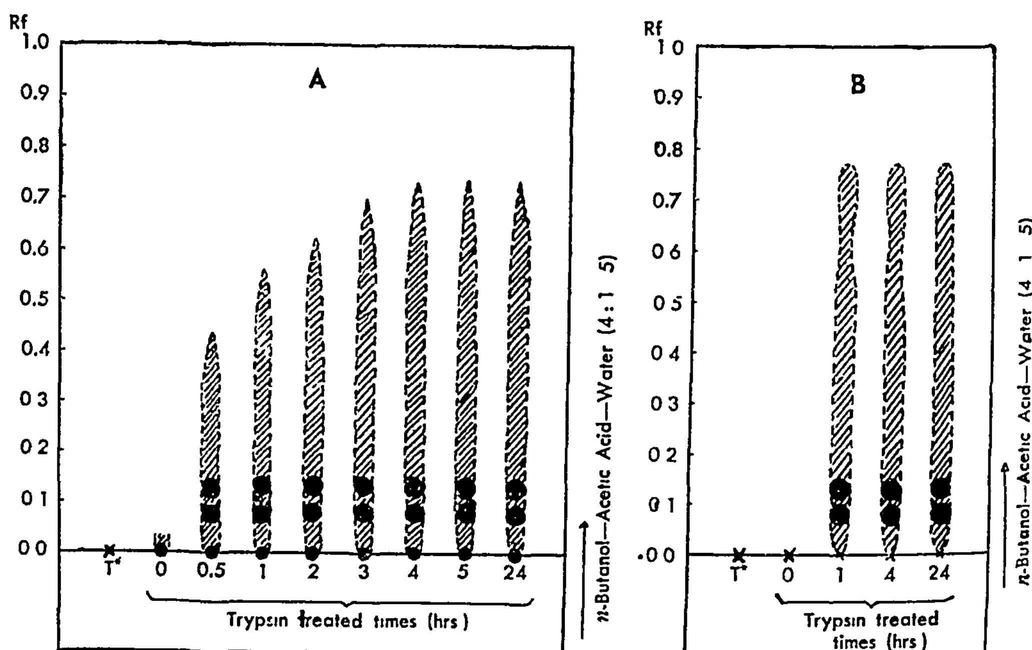


FIGURE 15 Paper partition chromatograms of parotin solution treated with trypsin (*A*) Parotin solution digested with trypsin, (*B*) supernatant solution of precipitate with trichloroacetic acid T*, trypsin solution (Reproduced with permission from *Endocrinologia Japonica* [11])

TABLE 7

INFLUENCE OF TREATMENT WITH CHYMOTRYPSIN ON CALCIUM ACTIVITY OF PAROTIN

Incubation time* (hr)		Percentage of serum calcium-decreasing activity† Mean ± S E
0	Parotin alone	11 49 ± 1 39
0	Chymotrypsin alone	1 08 ± 2 61
1	Parotin with chymotrypsin	8 97 ± 1 48
3	Parotin with chymotrypsin	9 35 ± 1 04
5	Parotin with chymotrypsin	10 26 ± 2 34
7	Parotin with chymotrypsin	9 53 ± 0 86

* Incubating temperature 37 ± 0 5° C at *p*H 8 0
† Dose of parotin, 1 mg /kg. intravenous injection; dose of chymotrypsin, 30 μg /kg intravenous injection.

localized in the spleens of group B rats ($p < 0.01$), as compared with group A or C rats. On the other hand, the value for the blood in group B rats was significantly higher ($p < 0\ 025$) than that for group A or C. No statistically significant difference in radioactivity in the kidney was found for the three groups ($p < 0.1$). The fact that intact parotin, in contrast to bovine serum albumin, was able to decrease the rate of localization of radioactivity suggested that pa-

R_f 0.63 to 0.76. In the case of labeled proteins incubated with several rat tissue homogenates, considerable radioactivity was observed in the areas that lay between R_f 0.02 and 0 76. In the area R_f 0.76 to 1.02, where radioactive iodide ion was developed, however, no appreciable amount of radioactivity was found.

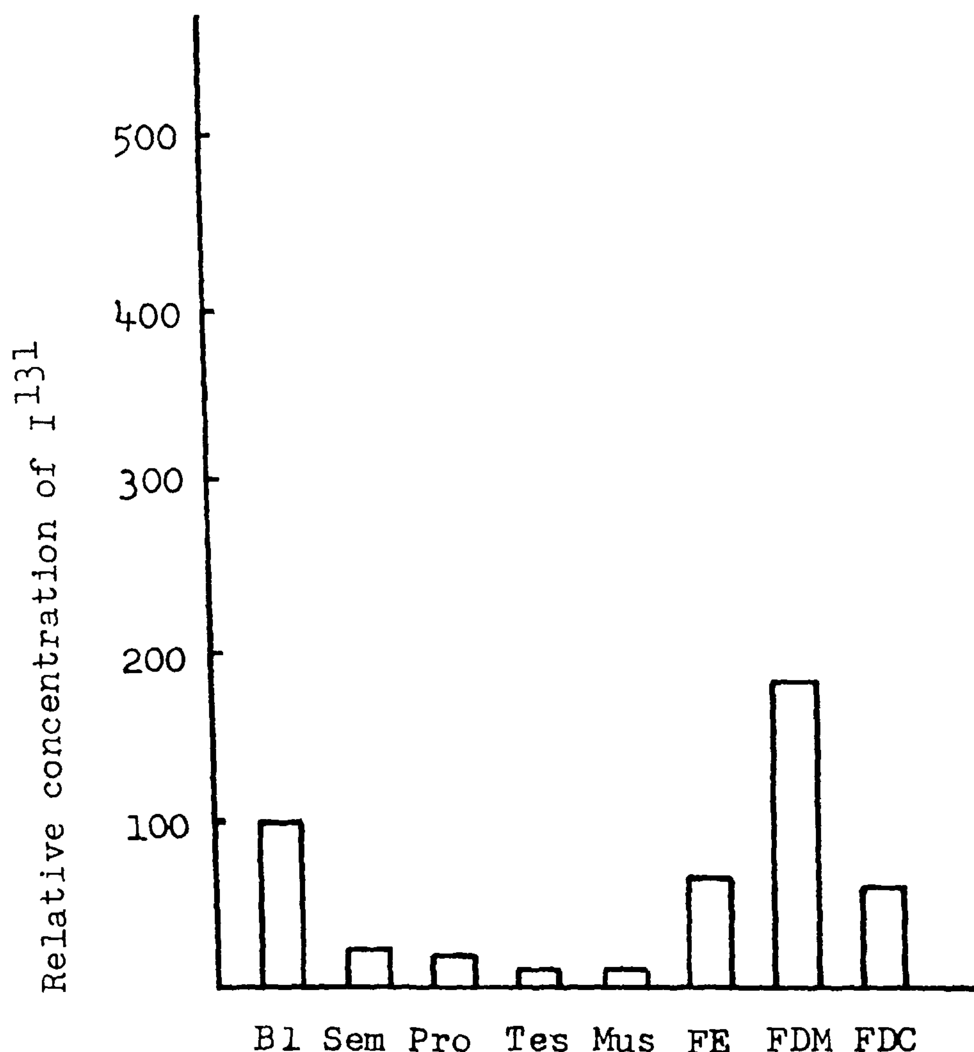


FIGURE 18 Distribution of radioactivity 20 min after intravenous injection of I^{131}-labeled parotin in rats Key. Bl, blood, Sem, seminal vescicle, Pro, prostate, Tes, testis; Mus, muscle, FE, femur epiphysis, FDM, femur diaphysis (marrow), FDC, femur diaphysis (cortex)

Concerning the direct deiodination of organic compounds, Kirkwood and his co-workers[20,21] reported the hypothesis that diiodotyrosine was deiodinated by salivary glands However, Chaikoff and his associates[22,23] opposed this theory.

Junqueira *et al.*[24] reported catheptic activity in the submaxillary gland; lysozymic activity in saliva has been observed by many workers[25-28] Recently Sreebny *et al*[28-31] and Shafer *et al.*[32-34] have reported on the characteristics of the proteolytic enzyme in the submaxillary gland and its relation to hypophysectomy and thyroxine or testosterone treatment. With regard to the na-

Degradation of I^{131}-Labeled Parotin in Vitro[16]

The degradation by several rat tissue homogenates of I^{131}-labeled parotin, as well as of similarly labeled bovine serum albumin, growth hormone, and casein, was studied. Measurements of the increase in radioactivity in the nonprotein fraction of the incubation mixture (FIGURE 24) showed that the labeled proteins,

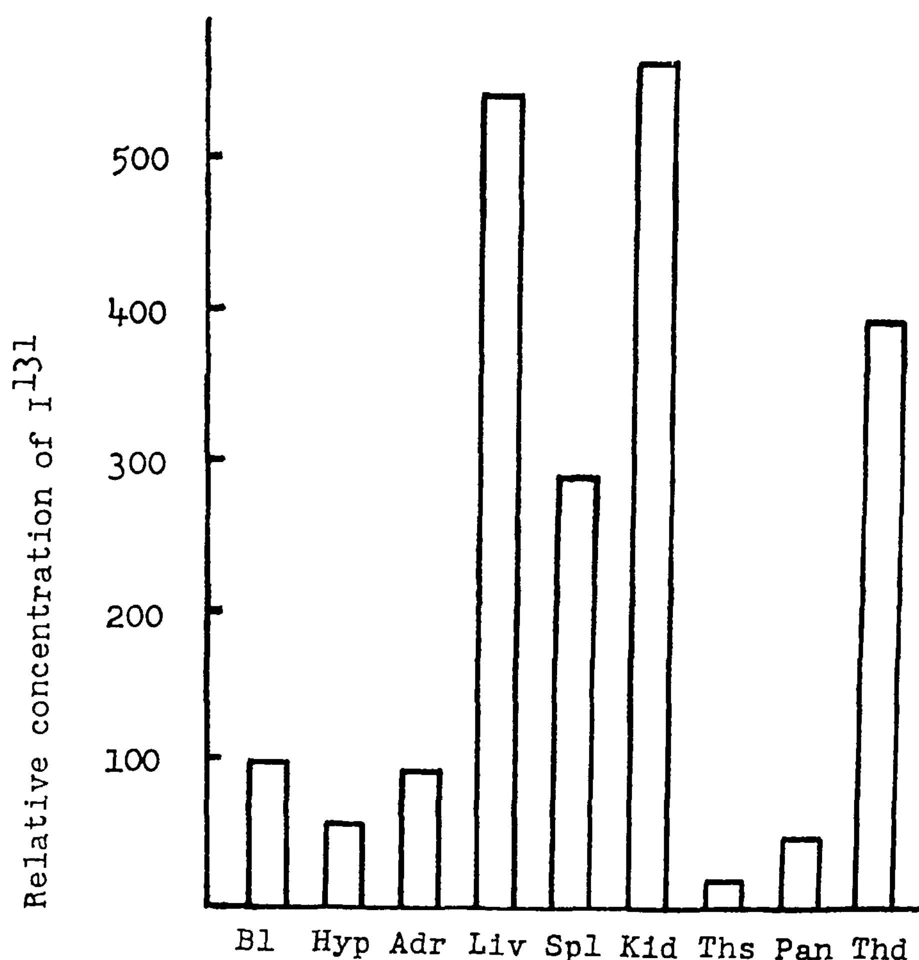


FIGURE 17 Distribution of radioactivity 20 min after intravenous injection of I^{131}-labeled parotin in rats Key: Bl, blood, Hyp, hypophysis, Adr, adrenal, Liv, liver, Spl, spleen; Kid, kidney; Ths, thymus; Pan, pancreas, Thd, thyroid

except for I^{131}-bovine serum albumin, were degraded with remarkable rapidity by submaxillary gland homogenate, while appreciable degradation was effected by other tissue homogenates.

The degradation of labeled proteins was thought to be the result of either proteolysis or direct deiodination. By quantitative (scintillation counter) radio-paper-chromatographic analysis, the former mechanism, namely, proteolytic degradation, was demonstrated (FIGURE 25). In the case of nonincubated I^{131}-proteins, almost all radioactivity was found around the original line (R_f −0.02 to 0.02), while iodide ion labeled with I^{131} appeared in the area from

lary extract and incubated liver extract showed very weak calcium activity or none at all (means of 3 rabbits: −8.15 and −8.10 per cent, respectively).

Biological Properties

Mesenchymal Tissues

Acceleration of the growth and calcification of cartilage tissues of tibia epiphysis, joint, nasal septum,[35] and alveolar bones was demonstrated histologically,

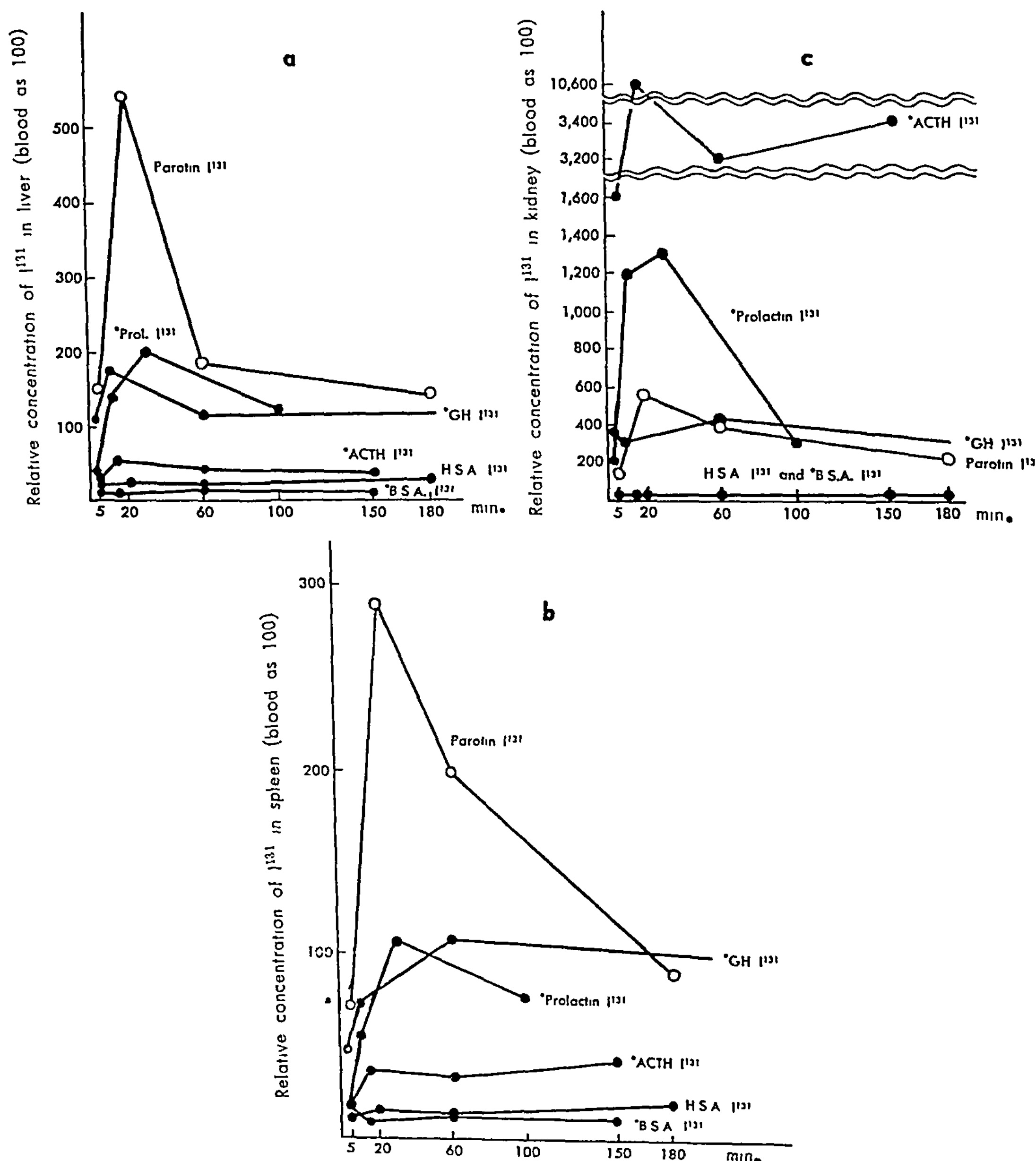


FIGURE 20 Comparison of I^{131}-labeled parotin with several other I^{131}-labeled proteins Key. Prol, prolactin, GH, growth hormone; HSA, horse serum albumin; BSA, bovine serum albumin; *, data of Sonenberg[16, 18, 19] Figure (*a*) liver, (*b*) spleen, (*c*) kidney (Reproduced with permission from *Endocrinologia Japonica*[12])

ture of the proteolytic enzyme or enzymes in the submaxillary gland, a series of experiments is in progress in my laboratory.

As will be described, my associate and I found a parotinlike substance of lower molecular weight (saliva parotin A) in human saliva. In this connection,

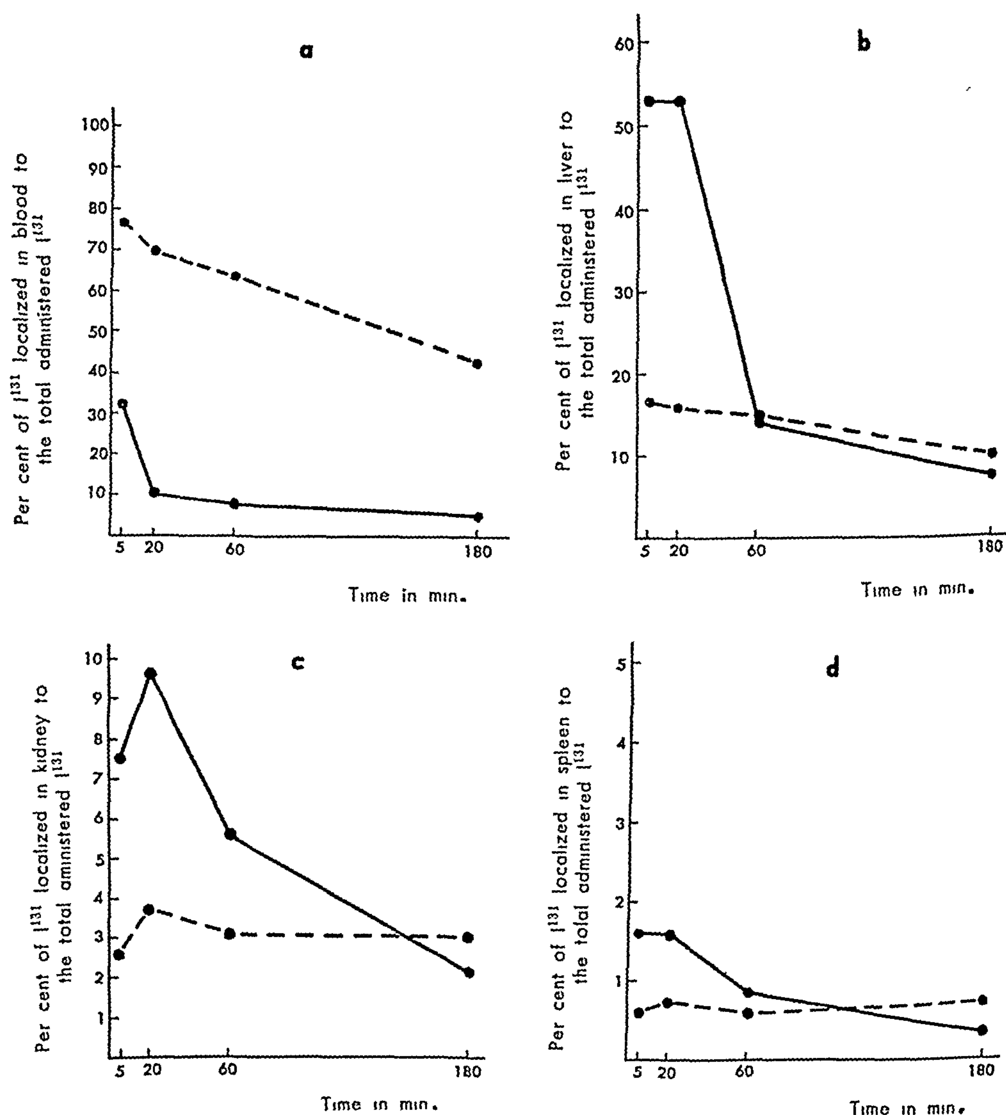


FIGURE 19 Localization of radioactivity in some organs after the administration of I^{131}-labeled parotin, ——, and I^{131}-labeled horse serum albumin, - - (*a*) Blood, (*b*) liver, (*c*) kidney, (*d*) spleen. (Reproduced with permission from *Endocrinologia Japonica*[12])

the strong proteolytic activity in the submaxillary gland is of interest with reference to the formation or secretion of biologically active principles at the salivary glands. Recently it was observed (Ito and Hirose, unpublished) that rat submaxillary extract, incubated at *p*H 7.6 and 37° C. for 2 hours and dialyzed against distilled water for 3 days, showed strong calcium activity (mean for 3 rabbits: −13.8 per cent), while similarly prepared nonincubated submaxil-

biochemically,[36] and also by a tracer experiment with radioisotopic phosphorus and calcium.[37]

H. Endo and I,[38] using tissue culture, demonstrated the effectiveness of parotin in promoting longitudinal growth and calcium deposition of bone, and suggested that parotin might directly stimulate some hard tissue development sites *in vivo*. The experiment was carried out as follows.

The femora of 7- or 9-day chick embryos were grown by the roller-tube

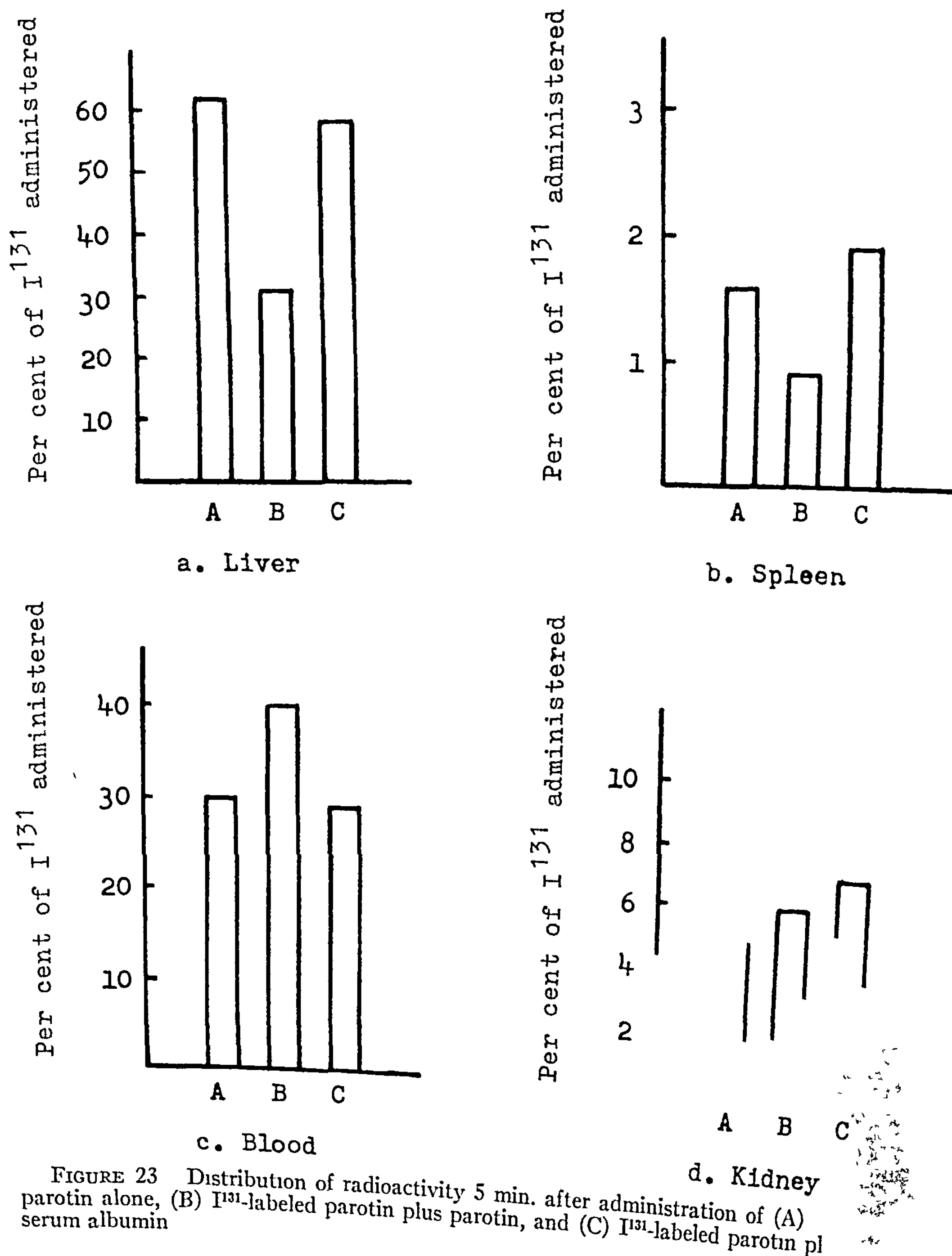


FIGURE 23 Distribution of radioactivity 5 min. after administration of (A) parotin alone, (B) I^{131}-labeled parotin plus parotin, and (C) I^{131}-labeled parotin pl serum albumin

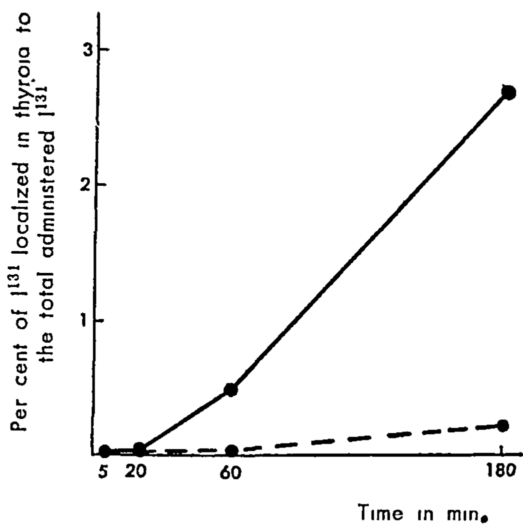


FIGURE 21 Localization of radioactivity in thyroid after administration of I^{131}-labeled parotin, ——, and I^{131}-labeled horse serum albumin, - - (Reproduced with permission from *Endocrinologia Japonica*.[12])

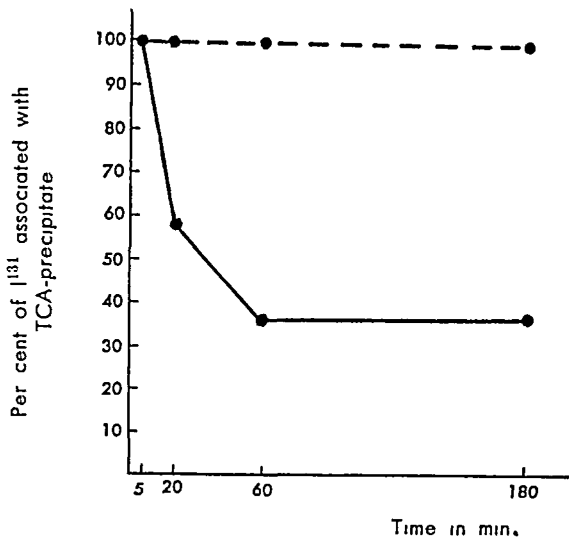


FIGURE 22 Results of trichloroacetic acid treatment of plasma after administration of I^{131}-labeled parotin, ——, and I^{131}-labeled horse serum albumin, - - (Reproduced with permission from *Endocrinologia Japonica* [12])

TABLE 8

DESIGN OF EXPERIMENT FOR DEMONSTRATING THAT ACCUMULATION OF I^{131}-LABELED PAROTIN IN LIVER AND SPLEEN IS CHARACTERISTIC OF PAROTIN ALSO

Groups	Number of rats	Injection
A	4	0 25 mg of I^{131}-parotin
B	4	0 25 mg of I^{131}-parotin 2 25 mg of parotin
C	4	0 25 mg of I^{131}-parotin 2.25 mg of B S A

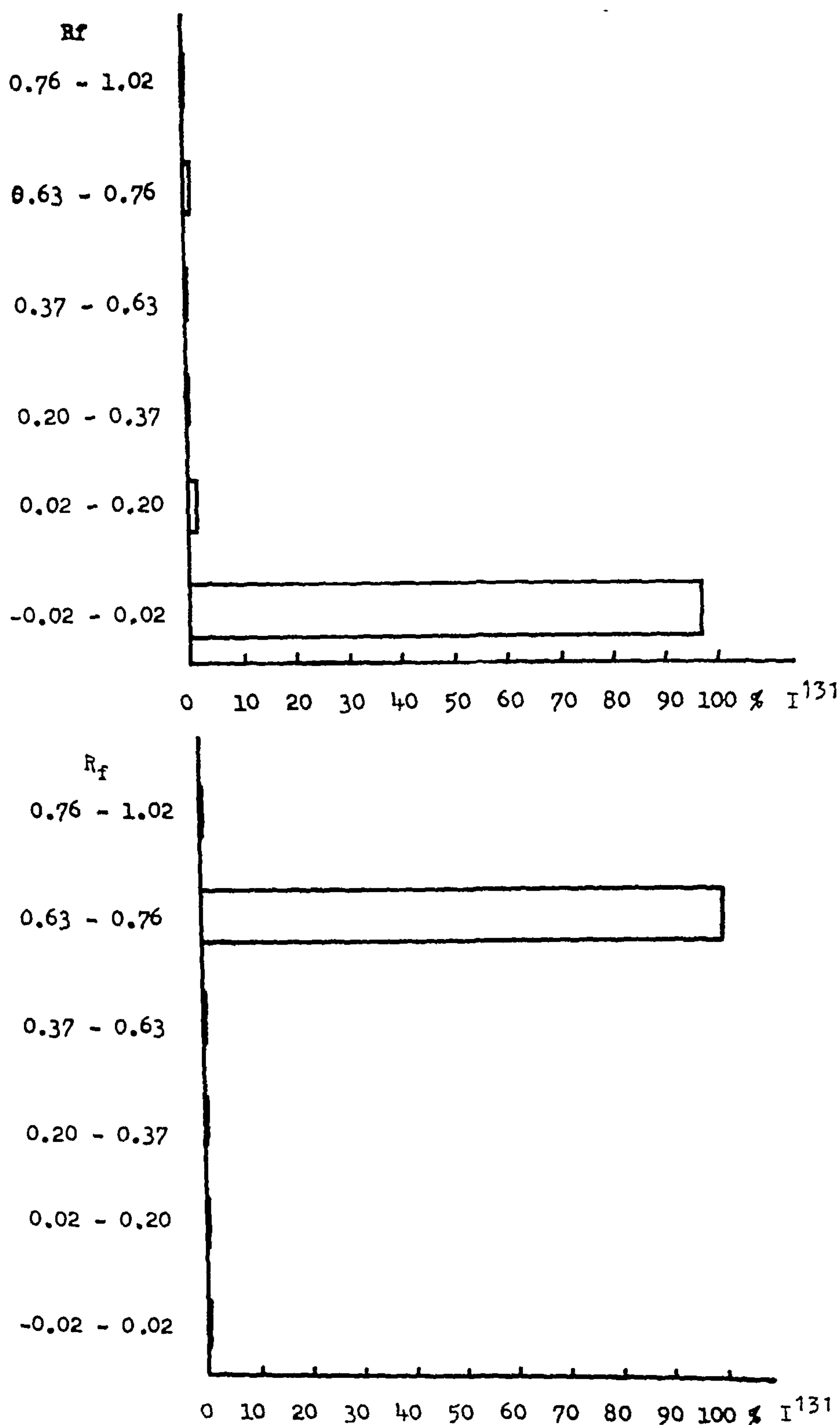


FIGURE 25 Typical results of quantitative radio-paper-chromatographic analysis of the mode of degradation of I^{131}-labeled proteins by several rat tissue homogenates. *Top:* Nonincubated I^{131}-parotin (control) *Bottom:* Iodine ion labeled with I^{131}.

culture method. No plasma was used; the culture medium consisted of 1 part of chick embryo extract, 2 parts of horse serum, and 7 parts of Gey's saline solution[39] The medium was renewed every other day. One femur was put in each tube containing 1 ml. of the medium. Throughout the ex-

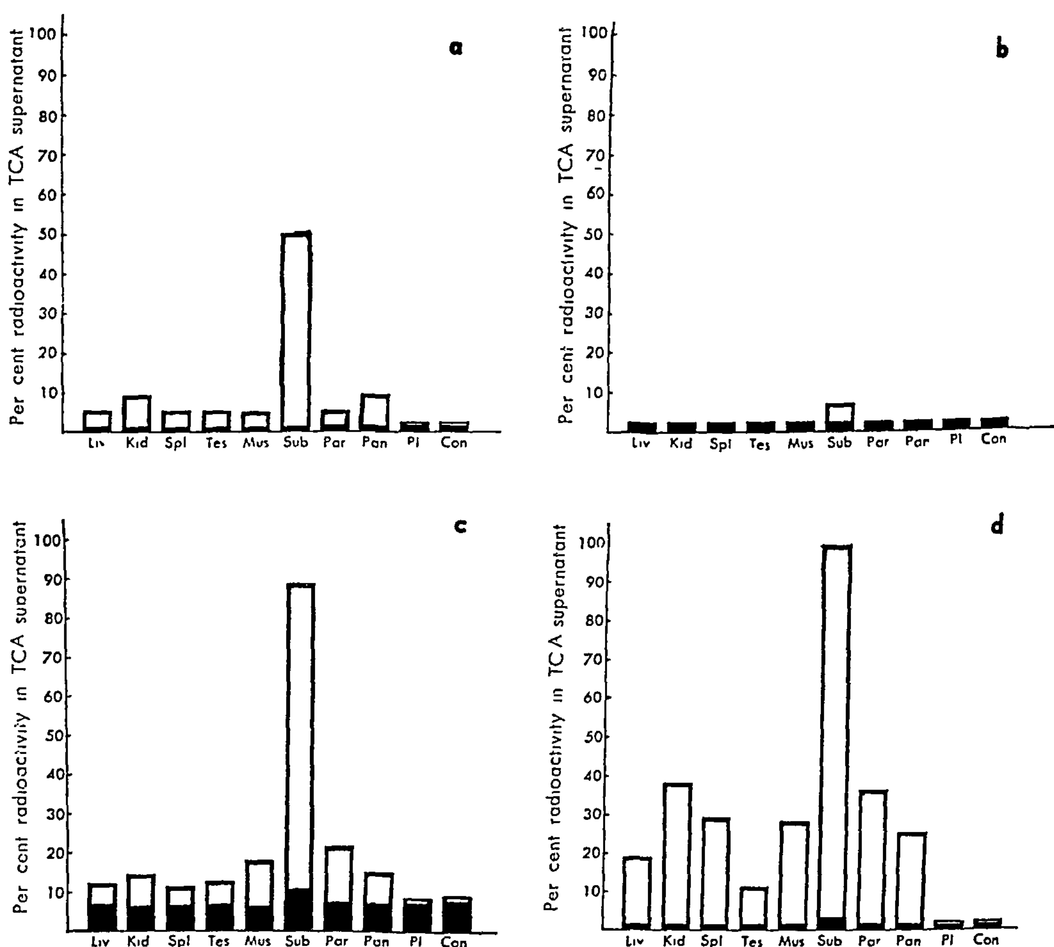


FIGURE 24 Degradation of I^{131}-labeled proteins 1 hour after incubation with several rat tissue homogenates Incubations were carried out with 1 ml of 7.5 per cent homogenate and 0 5 ml of substrate solution containing 45 μg. of I^{131}-labeled protein at 37° C and *p*H 7 4. Key· Liv, liver, Kid, kidney; Spl, spleen; Tes, testis, Mus, muscle, Sub, submaxillary gland; Par, parotid gland; Pan, pancreas, Pl, plasma; Con, control Light sections of graph represent nonincubated degradation. Figure (*a*) I^{131}-parotin, (*b*) I^{131}-bovine serum albumin, (*c*) I^{131}-growth hormone, and (*d*) I^{131}-casein (Reproduced with permission from *Endocrinologia Japonica* [15])

periments, the femora from one side of the chick embryo were cultivated in the normal medium and those from the opposite side of the same embryo in the medium containing the substance to be examined, in order to eliminate variation between embryos.

For the cultivation of bone, Gaillard[40] recommended a "dynamic medium" containing chick embryo extract prepared from embryos 2 or 3 days older than the cultivated tissues, to obtain rapid differentiation and vigorous growth

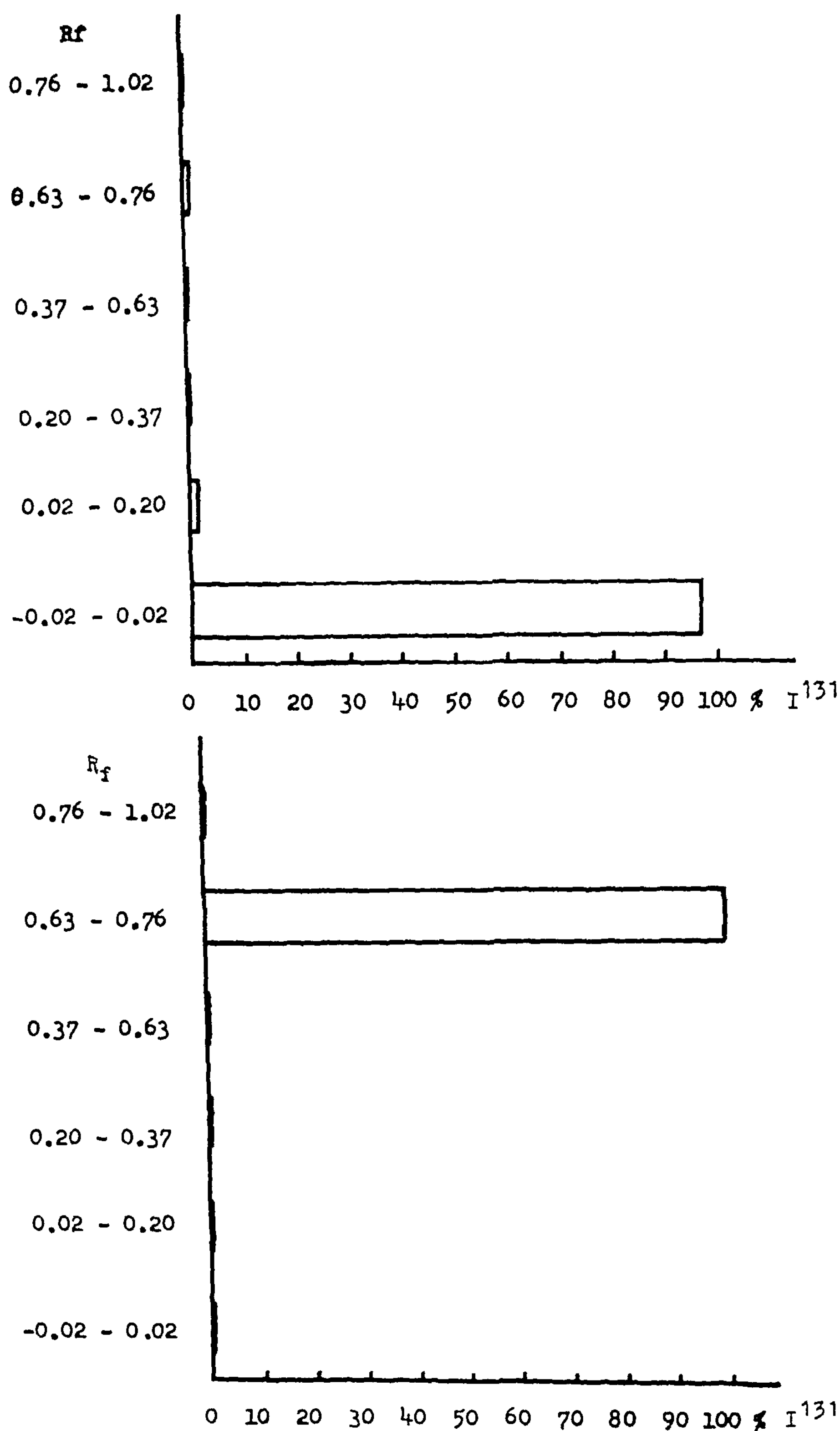
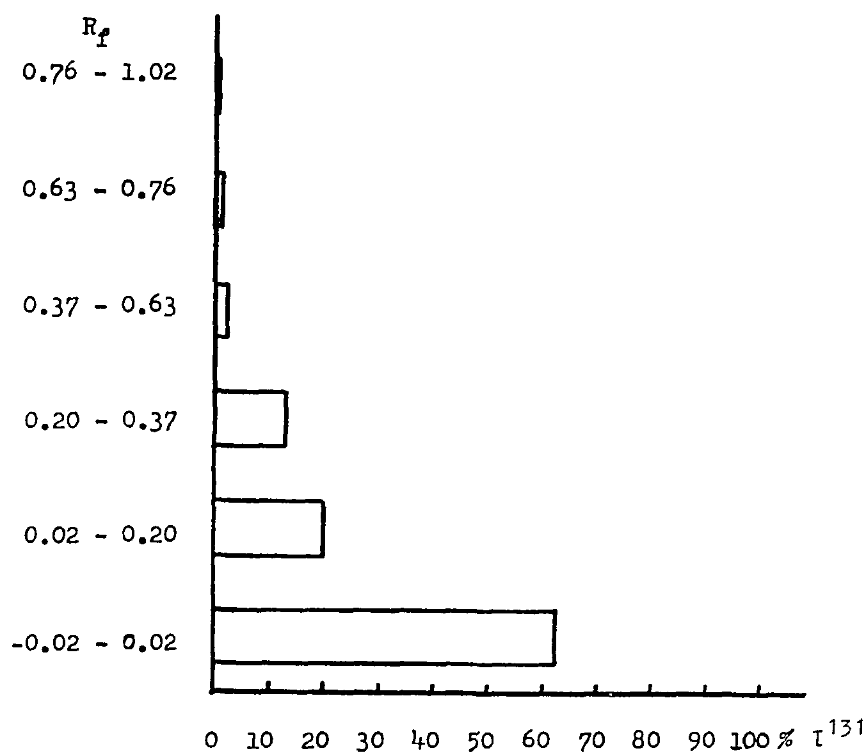


FIGURE 25. Typical results of quantitative radio-paper-chromatographic analysis of the mode of degradation of I^{131}-labeled proteins by several rat tissue homogenates *Top:* Nonincubated I^{131}-parotin (control) *Bottom:* Iodine ion labeled with I^{131}



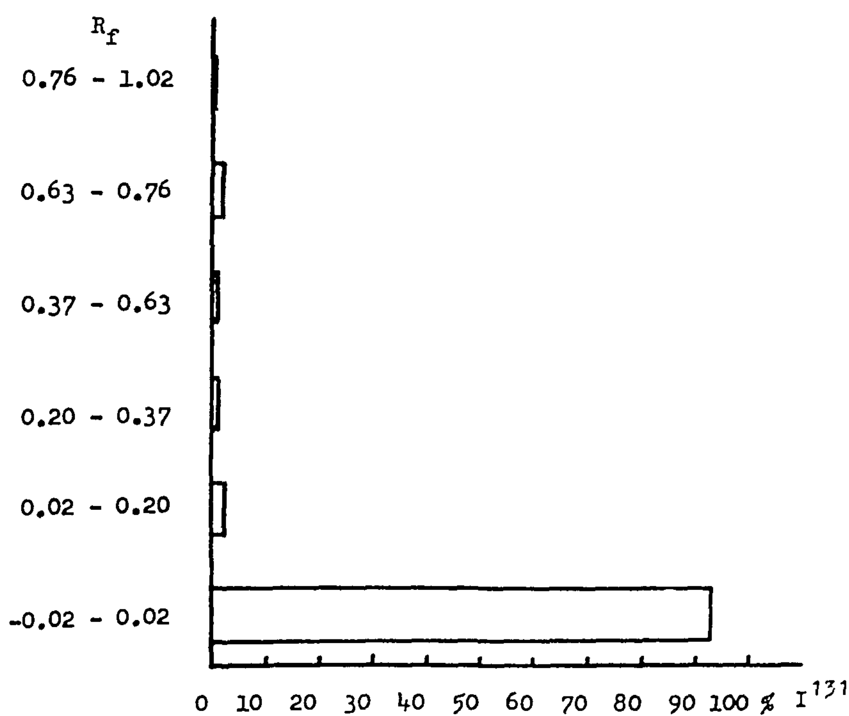
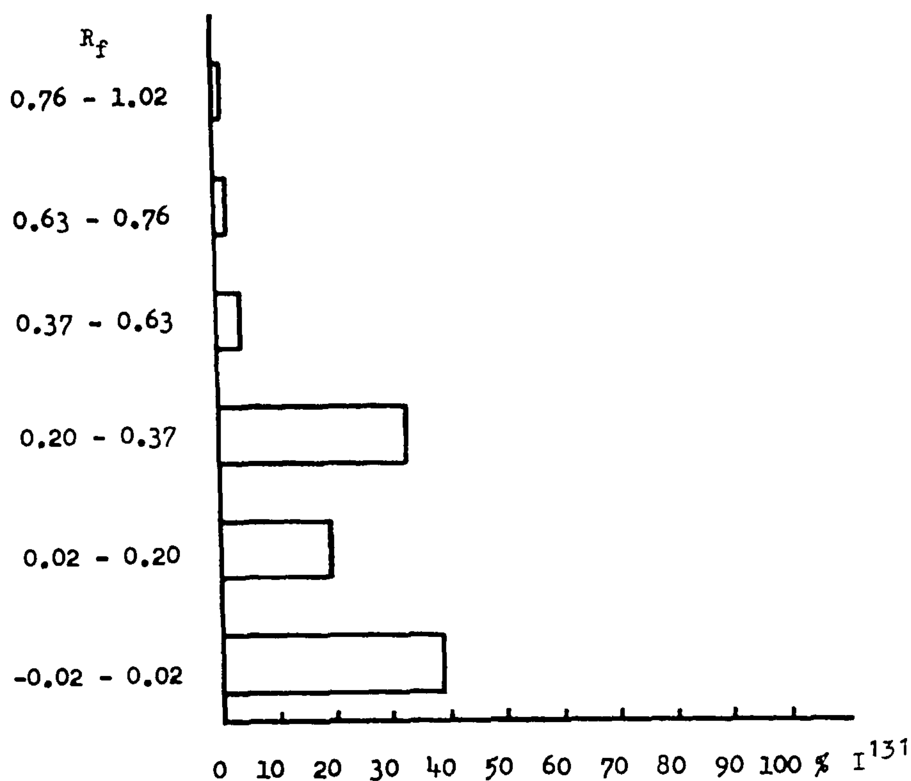


FIGURE 25. Results of radio-paper-chromatography *Top:* I^{131}-parotin incubated with submaxillary gland homogenate for 1 hour at 37° C. and *p*H 7.4 *Bottom:* I^{131}-parotin incubated with liver homogenate.



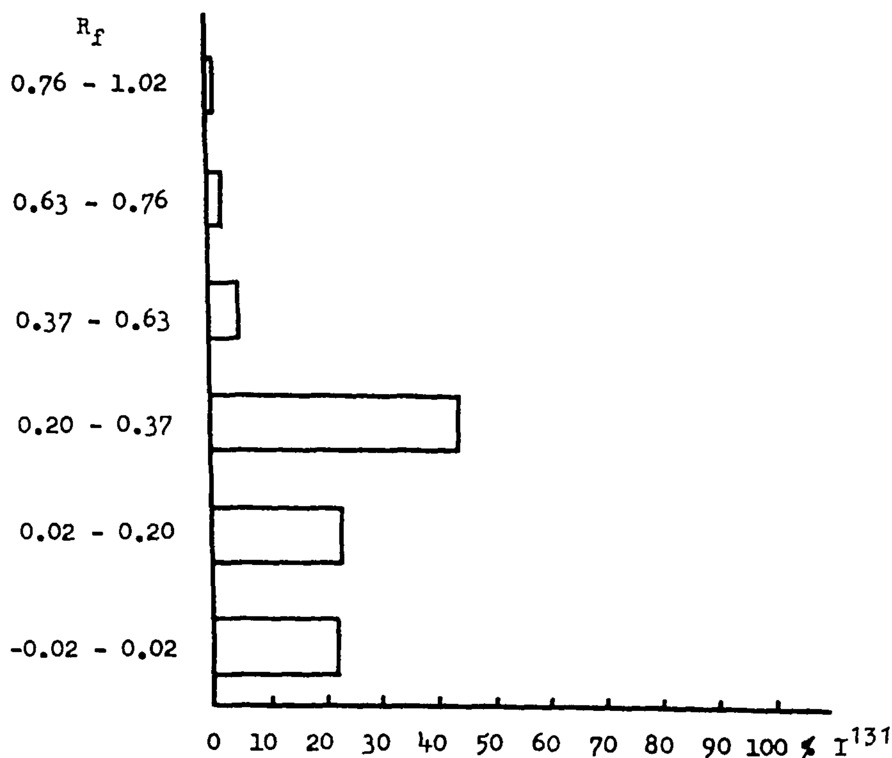


FIGURE 25. Results of radio-paper-chromatography *Top:* I^{131}-growth hormone incubated with submaxillary gland homogenate. *Bottom:* I^{131}-casein incubated with submaxillary gland homogenate.

in comparison with the normal medium. Miyazaki *et al.*[41] proved that chick embryo extract, prepared from embryos corresponding to the advancing age of the cultivated tissues, was essential for the higher ossification of chick embryo femur *in vitro*. Therefore, the chick embryo extract prepared in this manner was used in all experiments.

The length of the bones was measured every day with the ocular micrometer of a microscope. At the end of the experiments on 9-day chick embryo femora, 2 or 3 bones were combined and weighed in dry state. The calcium deposited was determined according to Sobel's acidimetric microtitration method,[42,43] slightly modified.

In the experiments on 7-day chick embryo femora, the stimulative effect of parotin on femora elongation was ascertained at a concentration of 140 γ/ml. of the medium as shown in FIGURE 26*c* ($p < 0.03$). The effect was reversed at a higher concentration, 280 γ/ml. (FIGURE 26*e*). However, since the elongation of the bones might not be a sufficient criterion for histologically and anatomically organized growth, the effects of parotin on calcification as well were examined in 9-day chick embryo femora, which normally would begin to calcify prior to hatching. The promotion of elongation of the explants was not found to be statistically significant, although a promotive tendency seemed to exist at a parotin concentration of 150 γ/ml (FIGURE 26*d*). On the other hand, egg albumin at the same concentration apparently did not affect the elongation (FIGURE 26*h*) or the calcium deposition of the bones (TABLE 10). The inhibitory effect was observed at the highest concentration, 300 γ/ml. ($p < 0.06$; FIGURE 26*f*). Perhaps this difference in the effectiveness of parotin on the longitudinal growth of 7-day and 9-day chick embryo femora results from the general fact that the younger the animal, the more effective is the substance added. As shown in TABLE 9, promotive or inhibitory effects on calcium deposition in 9-day femora were observed at concentrations where corresponding promotive or inhibitory effects were found in the elongation of 7- and 9-day femora. Therefore, it was suggested that parotin might act directly in some way on the growth of the bone

As already mentioned, parotin promotes calcification of incisor dentine in rats and rabbits. Fleming[44] has reported very recently on the effects of parotin on the growth centers of femora and incisors in mice as follows:

"Intramuscular injections of a parotid gland extract caused changes in the growth centers of mice femurs and in the zone of enamel secretion in the mandibular incisors. These changes were observed with 10 injections of parotin at 0.15 or 0.30 mg. for each injection. However, they were more extreme and took place sooner with the concentration of 0.30 mg. of parotin per injection. Growth and development of these mineralized areas were not enhanced. Changes in the enamel-secreting zone of the mandibular incisors were as follows: (1) granular appearance of the cytoplasm, thickening of cell walls of ameloblasts, (2) distortion of the ameloblastic Tomes's processes, (3) failure of ameloblasts to secrete compartmental protein arrangements in the orderly fashion, (4) distortion of the newly formed protein matrix, and (5) marked increase in vascularity of the area. Changes that took place in the growth centers of the femurs were as follows. (1) retardation of expected degeneration of cartilage cells, (2) decrease in proliferation of cartilage cells, (3) ballooning

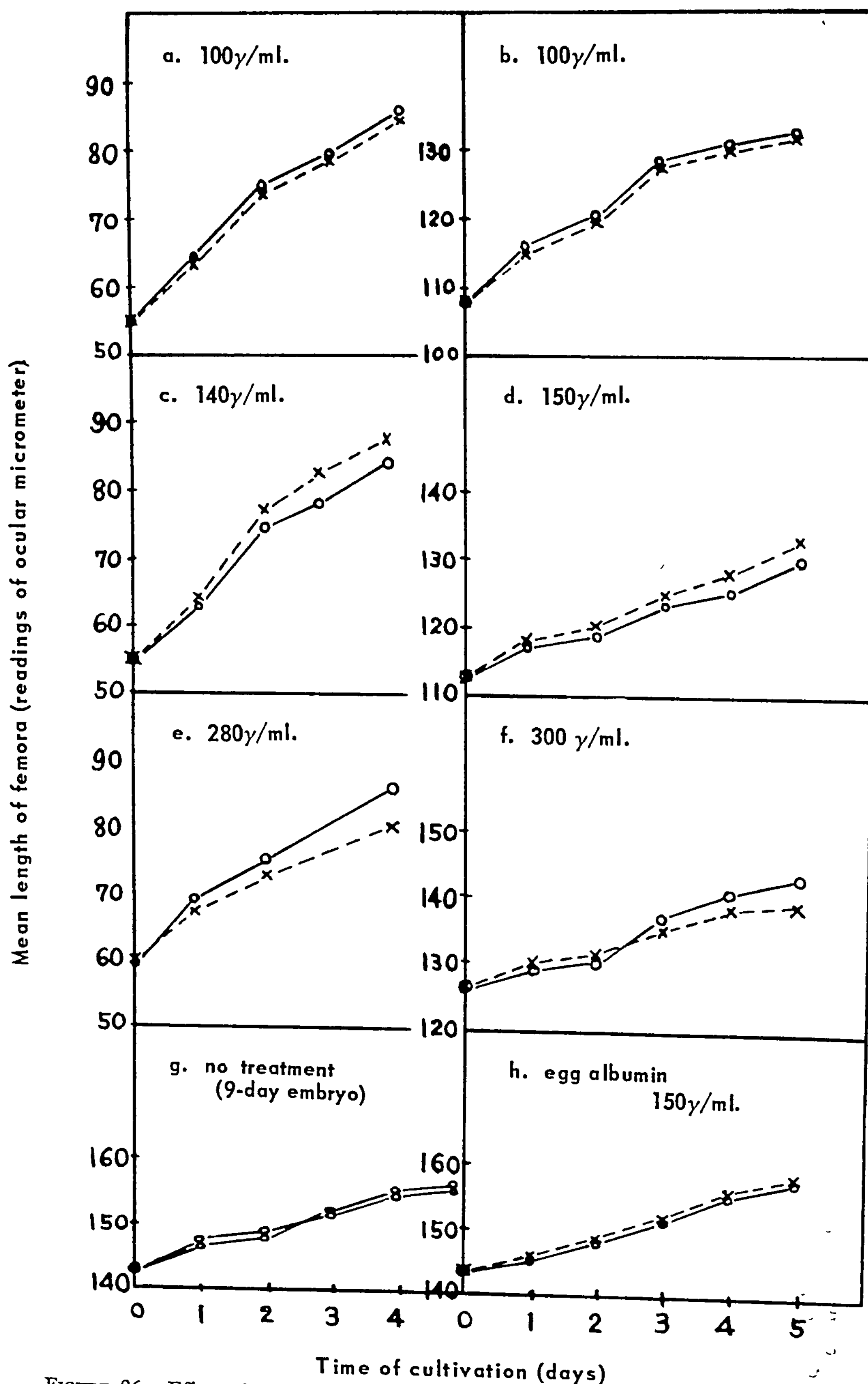


FIGURE 26. Effect of parotin on the longitudinal growth of 7- and 9-day chick embryo femora in tissue culture Mean length of femora of control group, O, and test group, X.

and/or reversion of cartilage cells, (4) osteoid proliferation deep within the epiphyseal cartilage, (5) increased numbers of blood vessels along the metaphyses, and (6) increase in cellularity and size of marrow spaces."

It has been shown by many Japanese workers that a small dose of parotin, for instance 0.3 mg /100 gm body weight injected intravenously in rats,

TABLE 9*
EFFECT OF PAROTIN ON CALCIUM DEPOSITION IN 9-DAY CHICK EMBRYO FEMORA IN TISSUE CULTURE

	I	II	III	IV	V	VI	Mean
Control							
Dry wt. of femora (mg.)	2 705	2.665	2 550	2.444	2.315	1.874	
Ca determined (μg)	76 5	55 4	63.3	58 1	53 7	55.4	
Ca (%)	2 83	2.08	2.48	2.38	2 32	2 96	2.51
150 γ/ml parotin							
Dry wt. of femora (mg)	2 755	2 416	2.480	2.321	2.230	1 735	
Ca determined (μg.)	73 0	58 9	64 9	63.3	56.5	56.2	
Ca (%)	2.65	2.44	2 62	2.73	2.53	3.24	2 70†
Control							
Dry wt. of femora (mg)	2.145	2.166	2 965				
Ca determined (μg)	84 1	91.5	115 3				
Ca (%)	3.92	4 22	3 89				
300 γ/ml. parotin							
Dry wt of femora (mg)	2 043	2.011	2 722				
Ca determined (μg.)	72 0	89 9	112 6				
Ca (%)	3.53	4.48	4.14				

* Reproduced by permission of *Endocrinologia Japonica* [38]
† Difference of means = 0 19, s^2 = 404 8; t = 2 313 ($p \approx 0 07$).

TABLE 10*
EFFECT OF EGG ALBUMIN ON CALCIUM DEPOSITION IN 9-DAY CHICK EMBRYO FEMORA IN TISSUE CULTURE

	I	II	III
Control			
Dry wt. of femora (mg)	2 683	2 546	3 710
Ca determined (μg.)	109.7	110 0	165 4
Ca (%)	4 09	4.32	4 46
150 γ/ml egg albumin			
Dry wt of femora (mg)	2 450	2 575	3 861
Ca determined (μg.)	107.4	111 5	165 4
Ca (%)	4 38	4 33	4.28

* Reproduced by permission from *Endocrinologia Japonica* [38]

resulted in the promotion of calcification in rat and rabbit incisors, acceleration of hair growth in rats, decrease of adrenal ascorbic acid in rats, and hypertrophy of the adrenal cortex, while doses about 10 times as large had opposite effects. In my opinion, the dose of parotin used in Fleming's experiment might have been so large that the effects of parotin were reversed: 10 intramuscular injections of 0.15 or 0.3 mg. were given every third day to mice weighing 25 gm

Okada[45] showed that parotin accelerated the development and growth of hair, using the Okada-Enaga thallium acetate injection time-marking method.

Mori[46] reported that parotin promoted the general growth, especially the growth of the bone tissue, of chick embryos *in ovo*. He surmised that a certain amount of calcium in the egg and the eggshell moved very easily to the embryo upon injection of parotin. He also reported that injection of small amounts of parotin corrected imperfect growth of chick embryos caused by the loading of calcium preparations.[47]

Takizawa[2] reported that parotin was effective in the maintenance and proliferation of elastic fiber systems in the aorta or the skin, in capillary blood

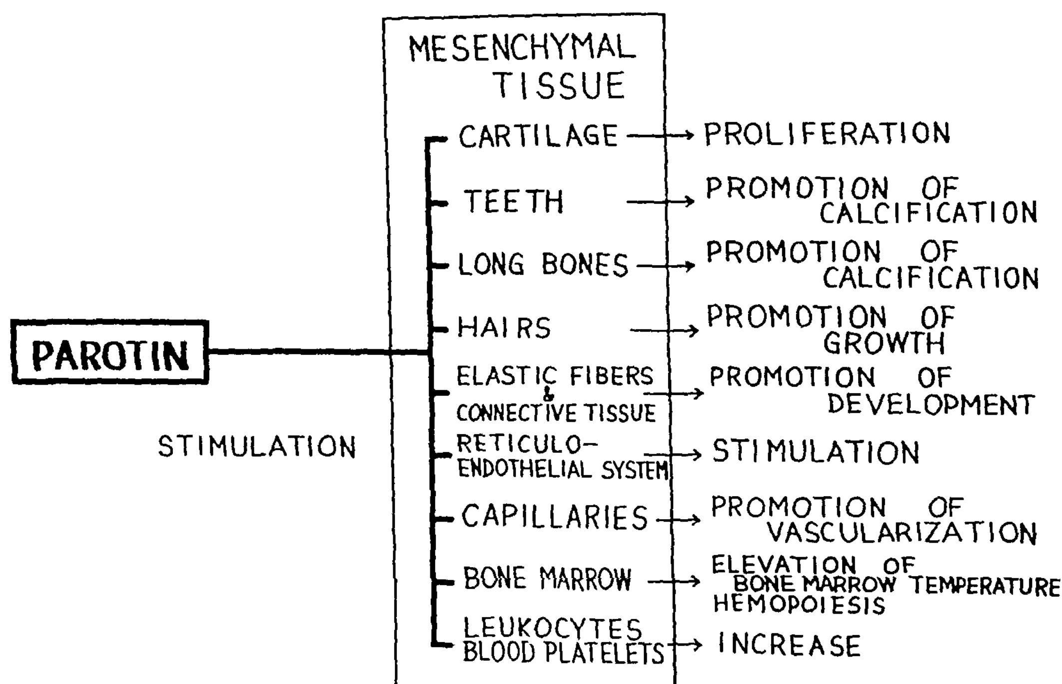


FIGURE 27. Schematic diagram of the effects of parotin on mesenchymal tissues

vessels in the skin, and in other mesenchymal tissues such as reticuloendothelial systems, connective tissues, and hematopoietic organs.

Mineral Metabolism

As already mentioned, parotin lowers the serum calcium level remarkably in rabbits. Similar effects have been observed in dogs and humans. The decrease of calcium has been shown to occur in the nondialyzable fraction, but not in the calcium ion-fraction of serum. Parotin is said to decrease the citrate in serum more remarkably than it decreases the calcium. Although parotin increases the serum magnesium level, the inorganic phosphate level in serum is not affected noticeably. The sodium and potassium chloride in serum increased transitorily, while in urine these decreased upon administration of parotin.

Protein Metabolism

S. Tsurufuji and I[48] found that the total protein in serum decreased remarkably after the injection of parotin in rabbits. Subsequently, Takaoka[4] and Yamaguchi[49] observed in rabbits and dogs that three months after removal of both parotid and submaxillary glands, the total amount of protein in serum had increased considerably; the protein ratio (the ratio of serum albumin to serum globulin) had become much smaller than that for the normal animals, while the administration of parotin to normal animals caused a conspicuous decrease in the total amount of protein in serum and an increase in the protein ratio. By electrophoretic analysis, these changes in the protein ratio accompanying asialadenism or following the injection of parotin were found to be due, respectively, to the decrease in albumin and the concurrent increase in α-globulin (and, probably, γ_1-globulin) or to the increase in albumin and the decrease in globulin. Furthermore, these investigators reported[4,49] that the intraperitoneal injection of parotin into rats decreased remarkably the total amount of nitrogen in urine, and that administration of parotin to rabbits caused a decrease in the total amount of nitrogen, a stoppage of creatine excretion in urine, a decrease in residual nitrogen in serum, and decreases in serum potassium and calcium.

Takaoka and his associates[50] observed that the oxygen consumption of rat liver homogenates increased upon injection of parotin and reached a maximum 6 hours after injection, and that this increase was inhibited by the addition of malonic acid to the homogenates. From these findings they concluded that parotin accelerated oxidation first in the tricarboxylic acid cycle and then in oxidative phosphorylation. Yuasa[51] observed that the P/O ratio in rat liver homogenates increased 6 hours after the injection of parotin; moreover, Sase[52] reported that parotin increased the P/O ratio in the mitochondria of hamsters and prevented the thyroxine-initiated decrease in the ratio. Parotin acts on the protein metabolism anabolically and inhibits the protein-catabolic action caused by thyroxine.

Yuasa[51] found that parotin did not affect the activity of liver alkaline phosphatase, but that it did decrease the activity of serum alkaline phosphatase and liver arginase and increased the activity of transaminase in liver and heart and cathepsin in liver. Ishikawa[53] observed that the incorporation of glycine-1-C^{14} into liver protein and the turnover rate of RNA-P in liver were both increased after the administration of parotin to rats From these findings Takaoka concluded that parotin promoted the synthesis of protein *in vivo.*

Carbohydrate Metabolism

The blood-sugar level was not affected by the injection of parotin to rabbits and dogs Shibata *et al.*[54] reported that the glucose tolerance of dogs was increased by the administration of parotin following glucose intake. The amount of pyruvate in blood was decreased by the administration of parotin to rabbits (Aonuma and Yoshimura[55]).

Lipid Metabolism

In experimental and clinical studies, Arai and Yagi[56] observed that parotin regulated the ratio of free cholesterol to total cholesterol in blood and also the

ratio of total cholesterol to total phospholipids in serum. After a single administration of 1 to 6 mg. of parotin to rabbits, the cholesterol level in blood increased, reaching a maximum 24 hours later, and then decreased to the initial value after a few days, while the total amount of phospholipids in serum increased remarkably. In the experimental hypercholesterolemia of rabbits, however, the total cholesterol decreased after a single administration of parotin and retained the lower value as long as one week after the injection.

Blood Pressure[57]

Parotin is not identical with kallikrein, since it does not affect blood pressure, respiration, or heart activity of phenobarbital-anesthetized rabbits, dogs, cats, or spinal cats given doses of 5, 10, or 20 mg. (FIGURE 28).

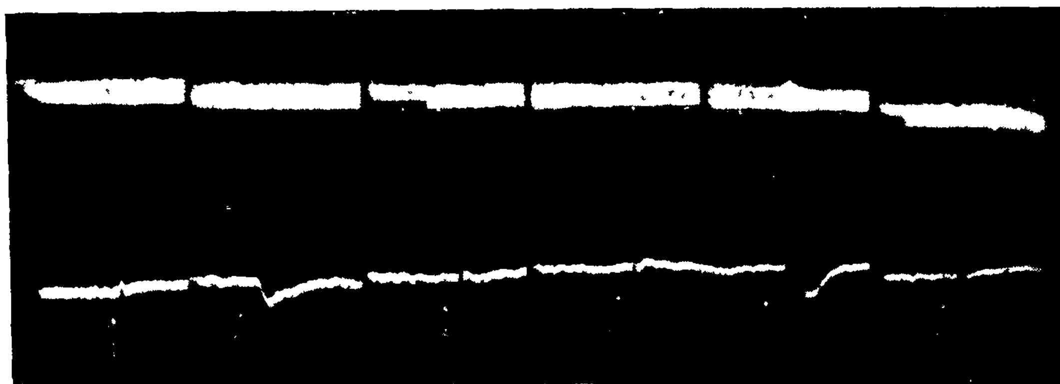


FIGURE 28 Effect of parotin on blood pressure (*lower curve*) and respiration (*upper curve*) of normal rabbits. Arrow 1, injection of parotin (20 mg per kg. of body weight); 2 kallikrein (0 5 U), 3,4,6, physiological saline solution (1 ml); 5, kallikrein (1 U).

Parotinlike Substances

S-Parotin in the Submaxillary Gland

According to the salivary gland endocrine theory, the principal role in the endocrine process is played by the parotid gland, and the submaxillary gland merely cooperates with it. Therefore, there must be an active substance in the submaxillary gland having the same effects as those of parotin. My associates and I[58-69] have succeeded in isolating parotinlike substances from bovine, equine, and hog submaxillary glands and have named them bovine S-parotin, equine S-parotin, and hog S-parotin, respectively. This purified S-parotin is considered to be one of the proofs of the salivary gland endocrine theory.

The bovine, equine, and hog submaxillary glands were treated similarly to the parotid gland from which crude parotin was isolated (FIGURE 29). The precipitate obtained at the isoelectric point, *p*H 4.5 (in the case of bovine parotid gland, *p*H 5.4), was submitted to fractional precipitation with ammonium sulfate, as was parotin. The precipitate obtained at 12.5 per cent ammonium sulfate was dialyzed against tap water.[59,60] In contrast to the case of parotin, in which the crude *p*H 5.4 precipitate and the 12.5 per cent

ammonium sulfate precipitate from the bovine parotid gland possessed only calcium activity and had no appreciable effect on the serum inorganic phosphate level, the corresponding fractions from the bovine or hog submaxillary glands were potent in lowering the serum inorganic phosphate level (hereafter this activity will be called phosphate activity), but were very weak in calcium activity. From the equine submaxillary gland two isoelectric precipitates, at *p*H 5.4 and *p*H 4.5, were obtained.

The separation of the phosphate activity from the calcium activity was effected by fractionation with ethanol at a low temperature.[61] The precipitate obtained at 12.5 per cent ammonium sulfate was dissolved in water at *p*H 8.0, and ethanol was added dropwise with cooling to the solution up to a concentration of 60 per cent. After separation of the precipitate, ethanol was

Bovine submaxillary glands (20 cattle) Acetone	Biological activities of main fraction*	
	Calcium activity (%)	Phosphate activity (%)
Powder (400 gm.) Dissolved in water (*p*H 8.0) Adjusted to *p*H 4.5.		
Precipitate (27.2 gm.)	6.40	10.13
Dissolved in water. Fractional precipitation with $(NH_4)_2SO_4$ (7 to 12.5 per cent).		
Precipitate (1.08 gm)	10.20	13.50
Dialyzed, fractionally precipitated with ethanol (60 to 80 per cent).		
Precipitate (0.163 gm.)	22.8	10.30
Dissolved in water, passed through alumina column, Amberlite IR-4B column, adjusted to *p*H 4 5, and the precipitate dialyzed and lyophilized.		
S-parotin (0.72 gm)	24.50	6.40

* Dose of 1 mg /kg. body weight.

FIGURE 29. Flow sheet of the isolation of bovine S-parotin from bovine submaxillary glands

added to the supernatant up to a concentration of 80 per cent, and the precipitate was separated. Calcium activity was found to be strong in the 80 per cent ethanol precipitate and weak in the 60 per cent ethanol precipitate. Phosphate activity was found to be slight in both precipitates. The 80 per cent ethanol precipitate was dissolved in water at *p*H 8.0 and passed through an alumina column. The filtrate was dialyzed against tap water and a saturated ammonium sulfate solution was added, up to a concentration of 12.5 per cent. The precipitate was dialyzed again and adjusted to *p*H 4.5. The resulting precipitate was dissolved in water at *p*H 8.0 and passed through Amberlite IR 4B. The filtrate was adjusted to *p*H 4.5, and the precipitate was lyophilized. This preparation (bovine S-parotin) showed high calcium activity (−24.5 per cent) and low phosphate activity (−6.4 per cent) upon intravenous injection of 1 mg./kg. into rabbits. Its purity was 93.8 per cent, by electrophoresis.

Each *p*H 5.4 and *p*H 4.5 precipitate from the equine submaxillary glands

was fractionated with ammonium sulfate in the same way, and it was found that the 12.5 per cent ammonium sulfate precipitate from the *p*H 5.4 precipitate contained principally calcium activity, while phosphate activity was concentrated in the 25 per cent ammonium sulfate precipitate from the *p*H 5.4 precipitate. The leukocyte activity of the former fraction in rabbits was similar to that of parotin and bovine S-parotin. Its purity was 74.1 per cent,

TABLE 11
COMPARISON OF SOME PHYSICOCHEMICAL PROPERTIES OF PAROTIN AND S-PAROTIN OBTAINED FROM DIFFERENT ANIMAL SOURCES*

S-parotin (% purity)†	Isoelectric point (*p*H)	Absorption maxima (mμ)	Halfwave potential of second wave in polarogram (volts)	
			Co(II)	Co(III)
Bovine parotin (ca 100)	5 7 (5.4)	277 ± 0.5	−1 35	−1 35
Bovine S-parotin (96 1)	4 5	276.5	−1.33	−1.35
Equine S-parotin (74 1)	5 4	276 0	—	—
Hog S-parotin (ca 100)	5.4	277 0	−1.35	−1.33

* Biological activities in calcium, leukocyte, and dentine-calcification activities are all equivalent
† Results of electrophoretic analysis

TABLE 12
COMPARISON OF AMINO ACIDS AND N-TERMINAL AMINO ACIDS FOUND IN PAROTIN AND S-PAROTIN OBTAINED FROM VARIOUS ANIMAL SOURCES

S-parotin (% purity)*	Nitrogen content (%)	Amino acids found in the hydrolyzates	N-Terminal amino acids
Bovine parotin (ca 100)	14 53	17 acids Ala, Arg, Asp, Cys, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Try, Tyr, Val	Ala, Asp, Gly
Bovine S-parotin (96 1)	14 05	16 acids· as above, less valine	Ala, Arg, His
Equine S-parotin (74 1)	10 11	16 acids as above, less valine	Gly, Met
Hog S-parotin (ca 100)	14 33	17 acids as above	Gly, Met

* Results of electrophoretic analysis

by electrophoresis. Results of coloring and precipitating tests for protein were positive, and the absorption maximum was 276 mμ.[65]

S-parotin obtained from bovine, equine, or hog submaxillary glands was equal not only to parotin in potency of calcium, leukocyte, and dentine-calcification activities and ability to raise bone marrow temperature, but also it had almost identical ultraviolet absorption spectra and polarograms (TABLE 11).

As shown in TABLE 12, the amino acids found in the hydrolyzates of S-parotin preparations from different animal sources were identical with those found in parotin, except that bovine and equine S-parotin were lacking in valine.

The N-terminal amino acids in S-parotin were different from those in parotin (alanine, aspartic acid, and glycine); alanine, arginine, and histidine occurred in bovine S-parotin; glycine and methionine, in equine and hog S-parotin.[65-68]

In the dried state, S-parotin containing as much as 10 per cent moisture can be stored for at least 43 months without loss of either calcium or leukocyte activities. In aqueous solution neither activity was influenced by acidic

TABLE 13*
INFLUENCE OF HEAT TREATMENT ON CALCIUM ACTIVITY OF S-PAROTIN

Treatment			Percentage of calcium-activity† (Mean ± S E)
*p*H	Temperature (°C)	Time (hr.)	
Control (untreated)			13 53 ± 0.22
8 0	60	3	10.88 ± 0 07
4 5	60	3	7.40 ± 0 66
4 5	100	0 5	9.68 ± 0.93

* Reproduced by permission from *Endocrinologia Japonica*.[19]
† Dose of S-parotin: 1 mg./2.5 kg. intravenous injection; mean value of 3 rabbits.

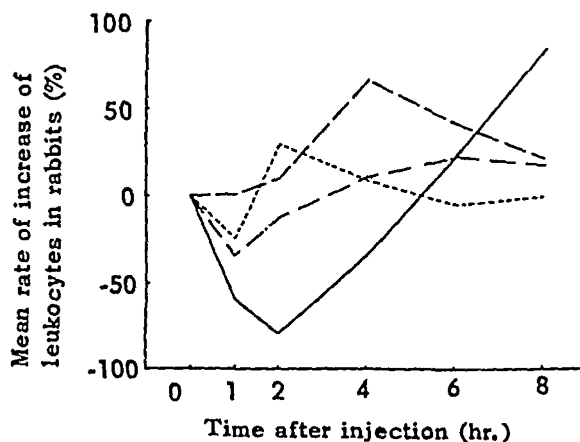


FIGURE 30. Influence of heat treatment of S-parotin on leukocyte activity Dose of S-parotin 1 mg./2 5 kg body weight in rabbits Key ——, control (untreated); – –, *p*H 8 0, 60° C , 3 hours; - -, *p*H 4 5, 60° C , 3 hours, — —, *p*H 4 5, 100° C , 0 5 hour (Reproduced with permission from *Endocrinologica Japonica* [69])

(*p*H 1.4) or alkaline (*p*H 11.8) treatment at 37° C. for 24 hours, in contrast to the results for parotin. When an aqueous solution of S-parotin was heated, the calcium activity was diminished slightly, while the leukocyte activity was destroyed completely (TABLE 13 and FIGURE 30). Upon treatment with urea or guanidine hydrochloride, the calcium activity of S-parotin was decreased, as it was in the case of parotin, while the leukocyte activity was diminished only slightly by treatment with guanidine hydrochloride Evidently, the leukocyte activity of S-parotin is more stable than the calcium activity against these denaturating reagents.[69]

It was determined that there is no radical solely responsible for the biologi-

cal activities of S-parotin; the free amino radical, the tyrosine phenyl radical, and disulfide bond are all responsible, just as in the case of parotin.[69]

Matsuda[70,71] reported that S-parotin injected as single doses of 0.5 mg./kg. body weight in intact and salivary gland-ectomized rabbits promoted the calcification of incisor dentine, and that the administration of doses 10 to 20 times as large inhibited calcification, as was found with parotin. Furthermore, he discovered the synergistic action of S-parotin and parotin on dentine-calcification activity: promotion of the calcification of the incisor dentine was evident after intravenous injection of 0.25 mg./kg. of parotin (one fourth the minimum effective dose) plus 0.05 mg /kg. of S-parotin (one sixth to one tenth the minimum effective dose). Karakasa[72] observed a similar synergistic effect on the bone and teeth in the salivary gland-ectomized rats.

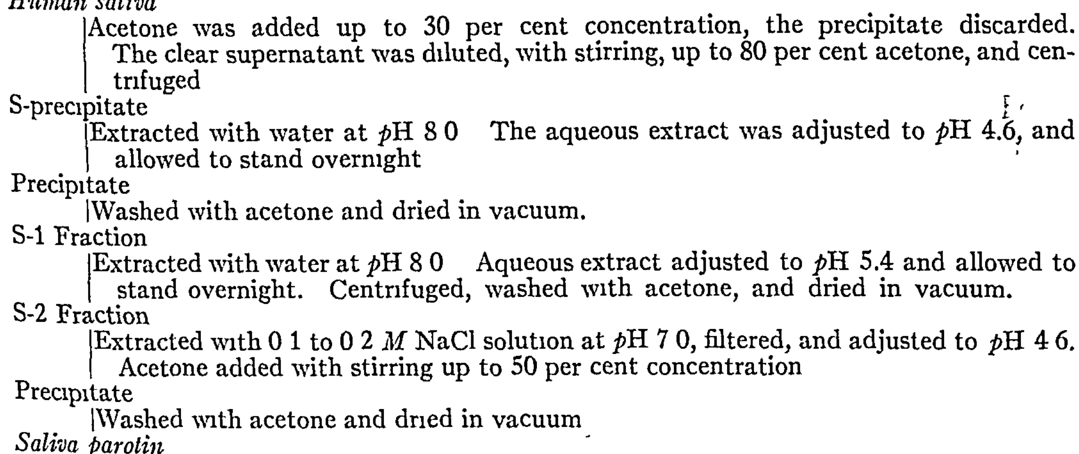

Human saliva
|Acetone was added up to 30 per cent concentration, the precipitate discarded. The clear supernatant was diluted, with stirring, up to 80 per cent acetone, and centrifuged
S-precipitate
|Extracted with water at *p*H 8.0 The aqueous extract was adjusted to *p*H 4.6, and allowed to stand overnight
Precipitate
|Washed with acetone and dried in vacuum.
S-1 Fraction
|Extracted with water at *p*H 8.0 Aqueous extract adjusted to *p*H 5.4 and allowed to stand overnight. Centrifuged, washed with acetone, and dried in vacuum.
S-2 Fraction
|Extracted with 0.1 to 0.2 *M* NaCl solution at *p*H 7.0, filtered, and adjusted to *p*H 4.6. Acetone added with stirring up to 50 per cent concentration
Precipitate
|Washed with acetone and dried in vacuum
Saliva parotin

FIGURE 31 Flow sheet of the isolation of saliva parotin from human saliva

PAROTINLIKE SUBSTANCES IN THE SALIVA

Saliva Parotin

Long ago T. Ogata discovered that the human saliva showed parotinlike activity when injected into experimental animals. A few years ago, S. Okabe and I succeeded in isolating a very potent fraction from human saliva, and named it saliva parotin[73]

Acetone was added to human saliva up to a concentration of 30 per cent, and the precipitate was discarded. To the supernatant solution more acetone was added, up to a concentration of 80 per cent. The precipitate, designated S-precipitate, was extracted with water at *p*H 8.0, and adjusted to *p*H 4.6. The material precipitated was washed with acetone, dried under reduced pressure, again extracted with water at *p*H 8.0, and adjusted to *p*H 5.4. The precipitate formed at *p*H 5.4 was extracted with 0.1 *M* sodium chloride at *p*H 7.0 and adjusted to *p*H 4.6. Acetone was added to this solution up to a concentration of 50 per cent. The precipitate, saliva parotin, was washed with acetone and dried *in vacuo* (FIGURE 31).

Saliva parotin is a white powder that dissolves readily in basic solution and

precipitates at *p*H 3.4. It contains 9.8 per cent nitrogen and is 72 7 per cent pure by electrophoretic measurement. The polarograms of S-parotin are similar to those of parotin, showing the characteristic 3 waves and a −1.36 volt half-wave potential of the second wave. S-parotin reacted positively to all color tests for protein, did not coagulate when heated in aqueous solution, but was precipitated by picric acid, trichloracetic acid, phosphotungstate, metaphosphate, sulfosalicylic acid, lead acetate, and lead sublimate solutions.

It was found by paper partition chromatography that the acid hydrolyzate of saliva parotin contained 16 amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. In addition, tryptophan was found in the alkaline hydrolyzate.

In rabbits, the biological activities of this preparation—calcium activity, leukocyte activity, dentine-calcification activity, and elevation of bone marrow temperature—are more potent than are the corresponding activities of parotin.

Saliva parotin differs from kallikrein, as shown by its lack of effect on the blood pressure of phenobarbital-anesthetized dogs.

The S-precipitates from the different sources of human saliva, for example, saliva from the parotid gland (A), saliva from the submaxillary and sublingual glands (B), and ordinary mixed saliva excreted from the above three glands (C), were compared with each other with respect to their calcium and leukocyte activities and yields. The results indicated that the biological activities were most potent in C and the weakest in A, and that the yields were greatest in A and least in B (Ito and Okabe, unpublished).

Saliva Parotin A

The method described above of separating saliva parotin from human saliva is disadvantageous because of variability in yield and biological activity, complexity, and difficulty of further purification. Therefore, a new method for purifying S-precipitate was developed that includes treatment with glacial acetic acid according to the method of Payne *et al*[74] for extracting ACTH-peptide from bovine hypophysis; by this method an extremely active parotinlike principle was obtained in the entirely homogeneous state. This new active principle was named saliva parotin A, in which "A" signifies treatment with acetic acid Although the biological activities are qualitatively the same as but quantitatively more potent than those of saliva parotin, the two substances are unlike in physicochemical and chemical properties

Isolation[75] The process is outlined in FIGURE 32. Four parts of acetone were added to one part of saliva from healthy adults, and the mixture was kept overnight. The precipitate (S-precipitate) was collected by centrifugation and dried *in vacuo* after being washed with acetone and ether The minimum dose of S-precipitate found to affect the calcium activity was 1.0 mg./kg. body weight, injected intravenously into rabbits

One part dried S-precipitate was added to 15 to 20 parts glacial acetic acid and extracted with stirring for 2 hours at 60° C. The extract was passed through a glass filter at room temperature. To the filtrate (F_1) was added saturated sodium chloride solution until the mixture became turbid, and then

an equal volume of ether was added. After being kept in the refrigerator overnight, the mixture was centrifuged; the precipitate (E-precipitate) was collected, washed with acetone and ether, and then dried *in vacuo*. The minimum dose of E-precipitate found to affect the calcium activity was 0.2 mg./kg., injected intravenously into rabbits.

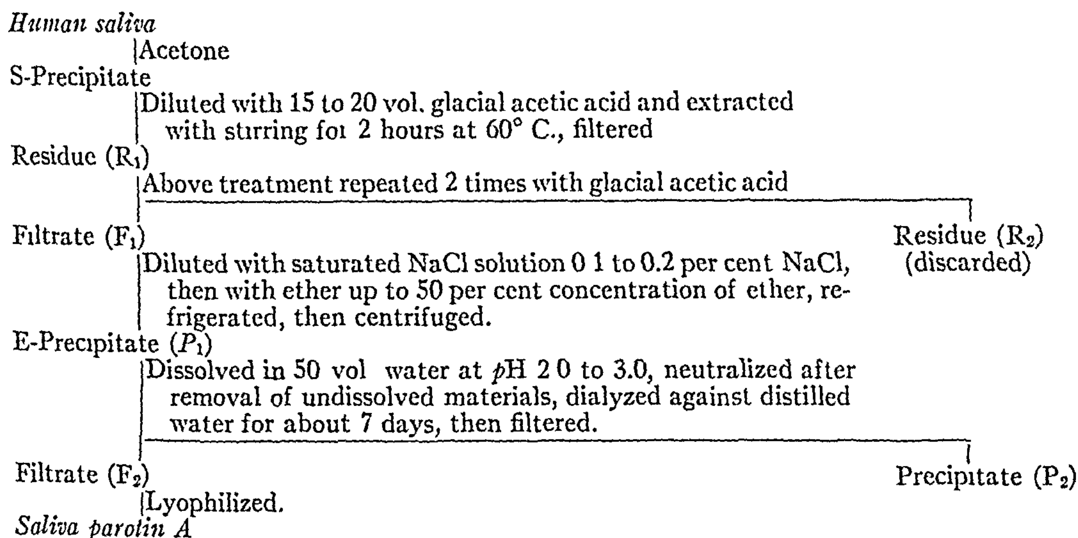


FIGURE 32. Flow sheet of the isolation of saliva parotin A from human saliva.

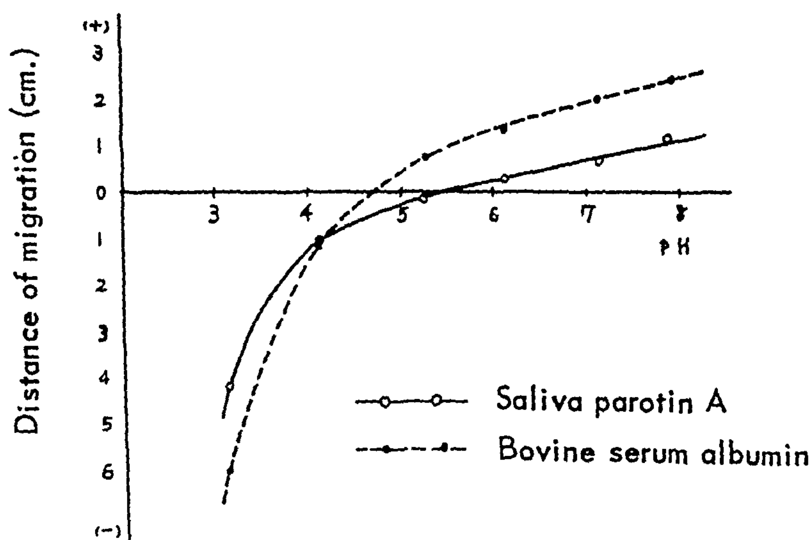


FIGURE 33 Estimation of the isoelectric point of saliva parotin A by paper electrophoresis

One part E-precipitate was dissolved in 50 to 60 parts water at *p*H 2.0 to 3.0 and dialyzed against distilled water. The precipitate that appeared during dialysis was discarded by filtration, and the filtrate (F_2) was lyophilized to yield 25 to 30 mg saliva parotin A per 100 ml. of saliva. The minimum dose found to affect the calcium activity was 0.1 mg./kg. body weight, injected intravenously into rabbits. This preparation can readily be employed for clinical purposes.

Physicochemical properties.[7] The isoelectric point of saliva parotin A was found to be *p*H 5.5 by paper electrophoresis in which bovine serum albumin was used as control and dextran for correction (FIGURE 33). Saliva parotin

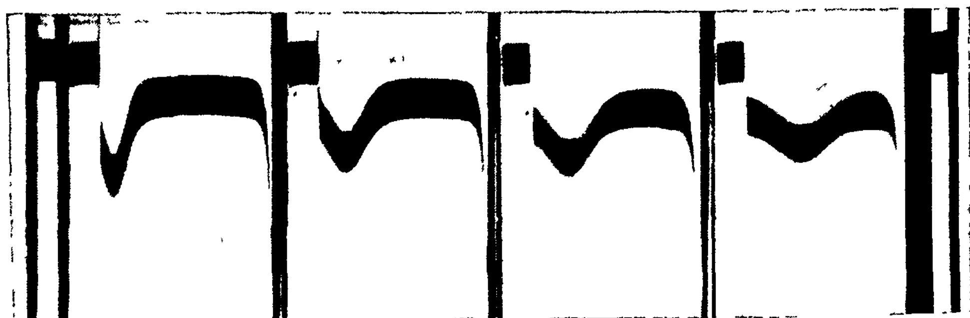


FIGURE 34. Ultracentrifugal patterns of saliva parotin A in Spinco Model E ultracentrifuge (10 per cent saliva parotin A in $\frac{1}{15}$ *M* phosphate buffer, *p*H 8.0, 59,780 rpm.) Right to left: 64, 128, 192, and 288 min.

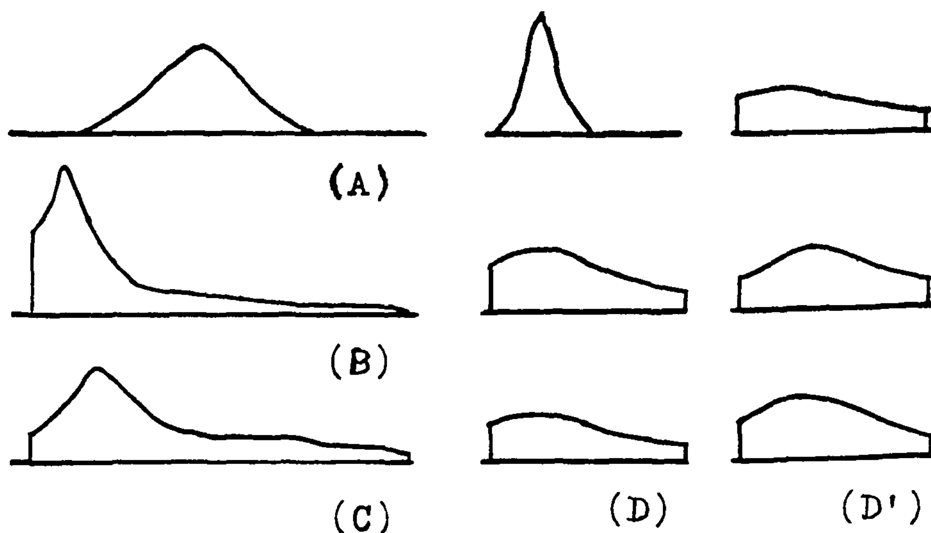


FIGURE 35 Electrophoretic patterns of saliva parotin A (A) 0.9 per cent in Veronal buffer, *p*H 8.6 (μ = 0.1), 7 mAmp., 185 v., 4° C., 150 min., (B) 1.0 per cent in phosphate buffer, *p*H 8.0, 8 mAmp., 150 v., 4° C., 90 min.; (C) 1.2 per cent in borate buffer, *p*H 8.8, 10 mAmp., 135 v., 4° C., 85 min., (D) 0.8 per cent in MacIlvane buffer, *p*H 7.7, 7 mAmp., 100 v., 4° C., 120 min. In each case, electrophoresis was applied for 90 to 120 min., but the main peak scarcely moved As seen in (D), when a MacIlvane buffer is used, the electrophoretic pattern is relatively flat That this phenomenon is not due to diffusion of the sample into the buffered solution was demonstrated by reversing the direction of the electric current after 120 min (D′).

A dissolved readily in water and all buffer solutions at any *p*H, and did not precipitate at the isoelectric point.

Ultracentrifugal patterns of a sample of saliva parotin A indicated homogeneity (FIGURE 34) The purity was calculated to be approximately 90 per cent by electrophoretic analysis with several types of buffer solutions (FIGURE 35). By paper electrophoresis, however, two fractions of saliva parotin A were distinguished, as shown in FIGURE 36.

Saliva parotin A showed an absorption maximum at 277.5 mμ and an extinction coefficient between 4 and 5 at that wave length (FIGURE 37).

The polarogram of saliva parotin A was quite different from that of parotin (FIGURE 38): the wave characteristic of protein was absent, and the reduction wave for cobaltous or cobaltic ions was observed.

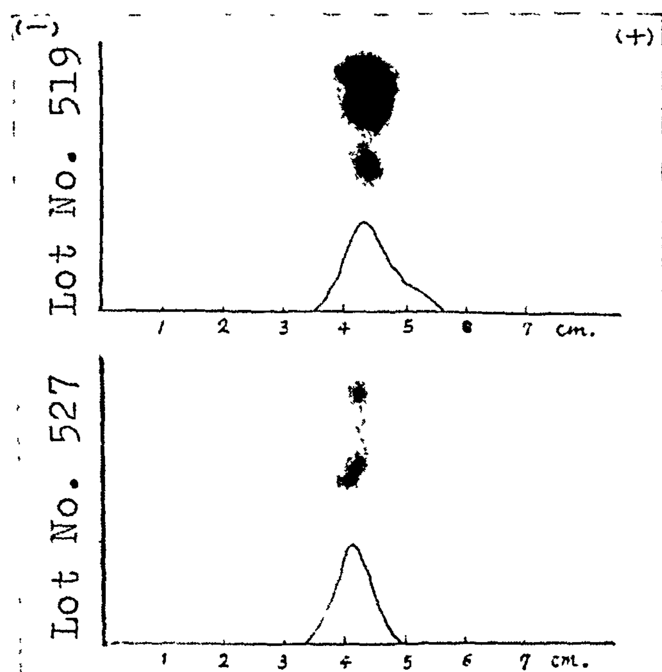


FIGURE 36 Paper electrophoretic patterns of samples of saliva parotin A Veronal buffer, pH 8.6 (μ = 0 1), 1 mAmp /cm. and 105 to 80 v for 6 hours, bromophenol blue stain

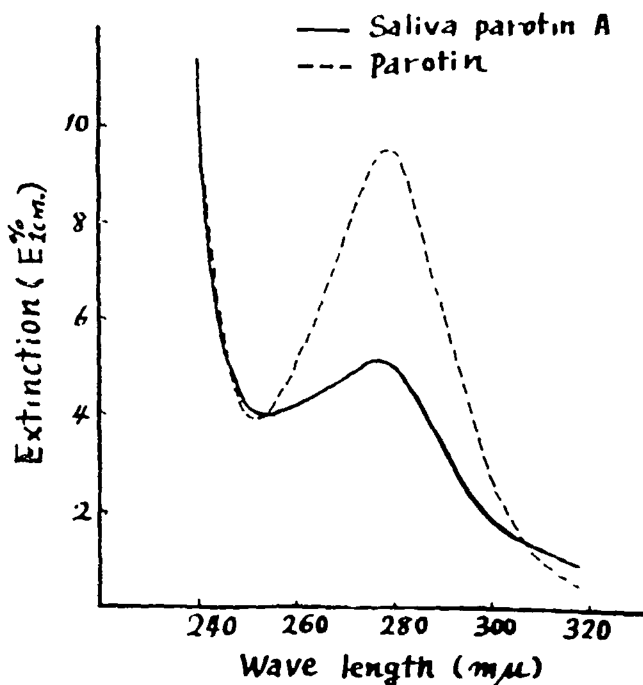


FIGURE 37 Ultraviolet absorption spectra of saliva parotin A and parotin

Chemical properties.[7] An aqueous solution of saliva parotin A gave positive results in biuret, Ninhydrin, Pauly's, xanthoprotein, Millon's, Sakaguchi's, and Molish's reactions. However, the results of Hopkine-Cole's and Liebermann's reactions were not distinct. Saliva parotin A precipitated in concentrated salt solutions, organic solvents, organic acids (for example, trichloroacetic acid, picric acid, and flavianic acid), and metaphosphoric acid.

The results of elementary analysis of saliva parotin A were as follows: C 46.62, H 6.99, and N 14.53 per cent. Sulfur and ash were negligible.

In the acid and alkaline hydrolyzates of saliva parotin A, 14 or 15 amino acids were found: alanine, aspartic acid, arginine, glutamic acid, glycine, histidine, lysine, leucine (and/or isoleucine) proline, phenylalanine, serine, tyrosine, valine, and tryptophan.

Biological activities.[7] The smallest dose of saliva parotin A found to affect calcium activity was 0 05 to 0.1 mg. per kg. body weight, injected intrave-

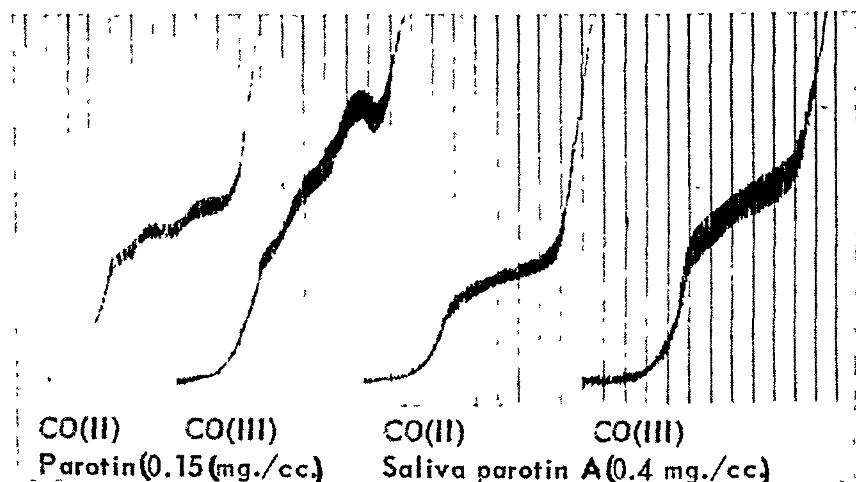


FIGURE 38 Polarogram of saliva parotin A and parotin

nously into rabbits (indicating a potency 10 to 20 times as great as that of parotin). As shown in FIGURE 39, the minimum calcium level was attained later, the larger the dose (0.02 mg./kg., 6 to 8 hours; 0.1 mg./kg., 24 hours; 0.2 mg /kg , 30 hours). When 5 to 10 times the minimum effective dose was injected, the percentage of decrease became irregular, and occasionally secondary effects such as fatigue and anorexia were observed.

As shown in FIGURES 40 and 41, the smallest dose of saliva parotin A found to affect leukocyte activity was 5 to 10 μg /kg. body weight, injected intravenously in rabbits (one one-hundredth to one two-hundredth the minimum effective dose of parotin). The leukocyte decrease persisted longer as the dose was increased.

Saliva parotin A, as well as parotin and other parotinlike substances, accelerated calcification of incisor dentine in rabbits, and was the most effective of them (the minimum effective dose being 5 to 10 μg./kg body weight, by single intravenous injection, FIGURE 4*a*). A dose 10 times as great as the optimal dose, however, inhibited calcification (FIGURE 4*b*).

Localization of I^{131}-labeled saliva parotin A.[76] Saliva parotin A was labeled with radioactive iodine by a method similar to that used for parotin. The biological activity of I^{131}-labeled saliva parotin A was largely retained (TABLE 22 and FIGURE 54). As shown in FIGURE 42*a*, 5 and 20 min. after injection of

Dose (mg./kg.)	Hours after injection 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30	Serum Calcium Decrease* (%)	S.E.
0.02		11.64 ±	1.62
		11.95 ±	3.92
0.1		14.53 ±	1.87
		17.19 ±	1.38
0.2		19.16 ±	4.15
		22.56 ±	4.31
		23.26 ±	2.52
		28.69 ±	2.87
Control		3.84 ±	1.27

*Mean rate for 3 to 5 rabbits.

FIGURE 39. Relationship between dosage and time at which serum calcium is minimal, following intravenous injection of saliva parotin A in rabbits Shaded areas correspond to times at which blood was drawn; horizontal lines correspond to observation of minimal values of serum calcium

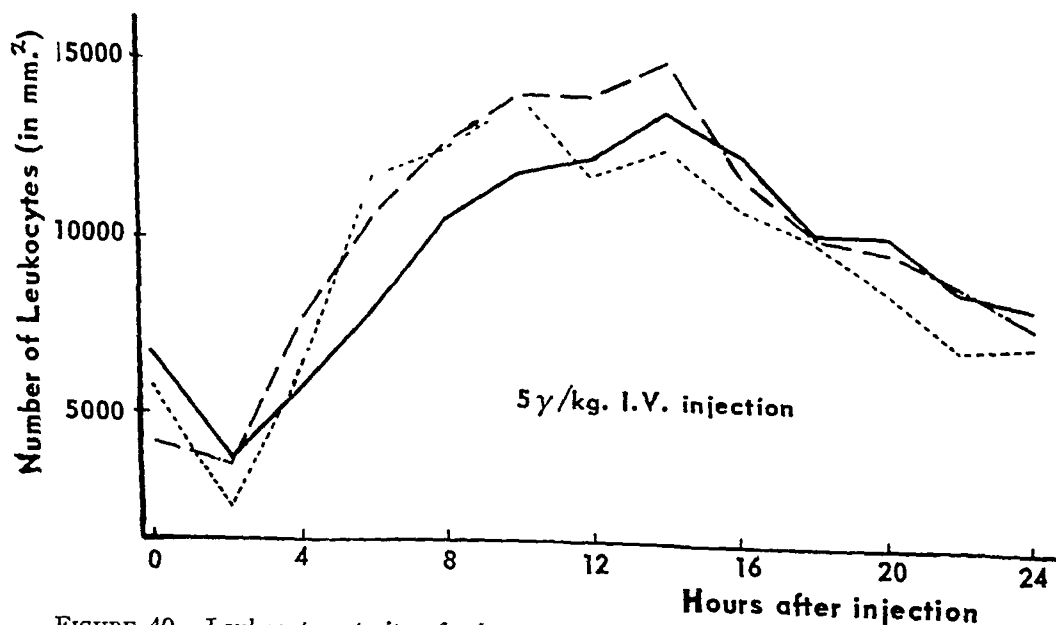


FIGURE 40 Leukocyte activity of saliva parotin A injected intravenously (5 μg /kg) in rabbits

labeled saliva parotin A into rats more than 30 per cent of the total radioactivity was localized in the kidney; in the case of parotin, less than 10 per cent of the radioactivity was found in this organ. This remarkably large amount of radioactivity was concentrated predominantly in the cortex (TABLE 14). The amounts of radioactivity localized in the liver and spleen were very small, compared with these amounts in the case of parotin (FIGURE 42*b* and *c*).

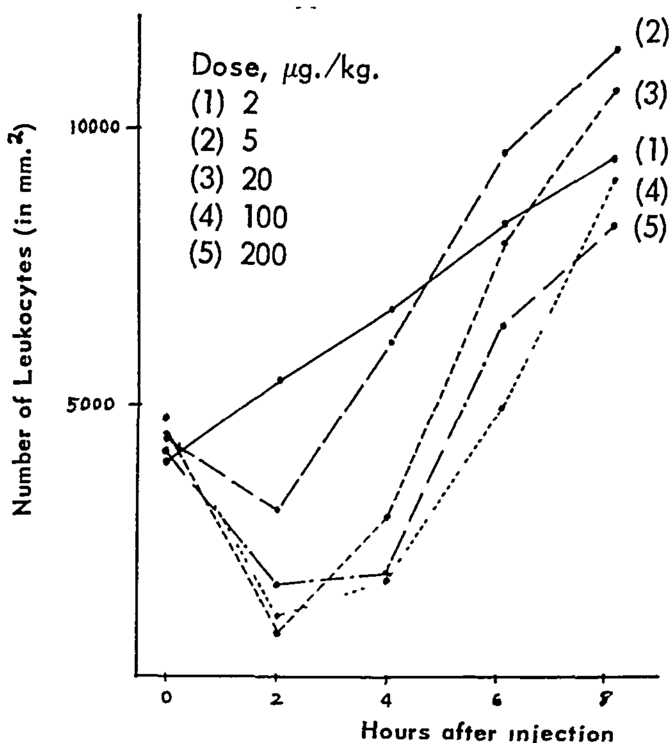


FIGURE 41. Leukocyte activity of saliva parotin A.

Purified Saliva Parotin A

Although saliva parotin A had very potent biological activities it was not completely homogeneous, and therefore further purification was undertaken Both fractional precipitation with trichloroacetic acid or ammonium sulfate and adsorption on oxycellulose were found to disperse the activity throughout all fractions.[77] However, fractionation with ethanol and ultracentrifugation (Spinco Model E ultracentrifuge) yielded almost homogeneous saliva parotin A (TABLES 15 and 16).

As shown in FIGURE 43, an equal volume of ethanol was added to a 1 per cent solution of saliva parotin A in an ice bath The solution was kept overnight in a refrigerator, and the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed. A precipitate that deposited in the cellophane tube was eliminated by filtration. The filtrate was diluted with distilled water to 1 per cent concentration and ultracentrifuged for 4 to 5 hours at 54,000 rpm. The lower layer of each tube was collected and lyophilized to

yield purified saliva parotin A. The over-all yield of purified saliva parotin A was 2 to 3 mg./100 ml. of human saliva.

Physicochemical properties. Purified saliva parotin A was shown to be nearly homogeneous, by electrophoretic and ultracentrifugal analyses (FIGURES

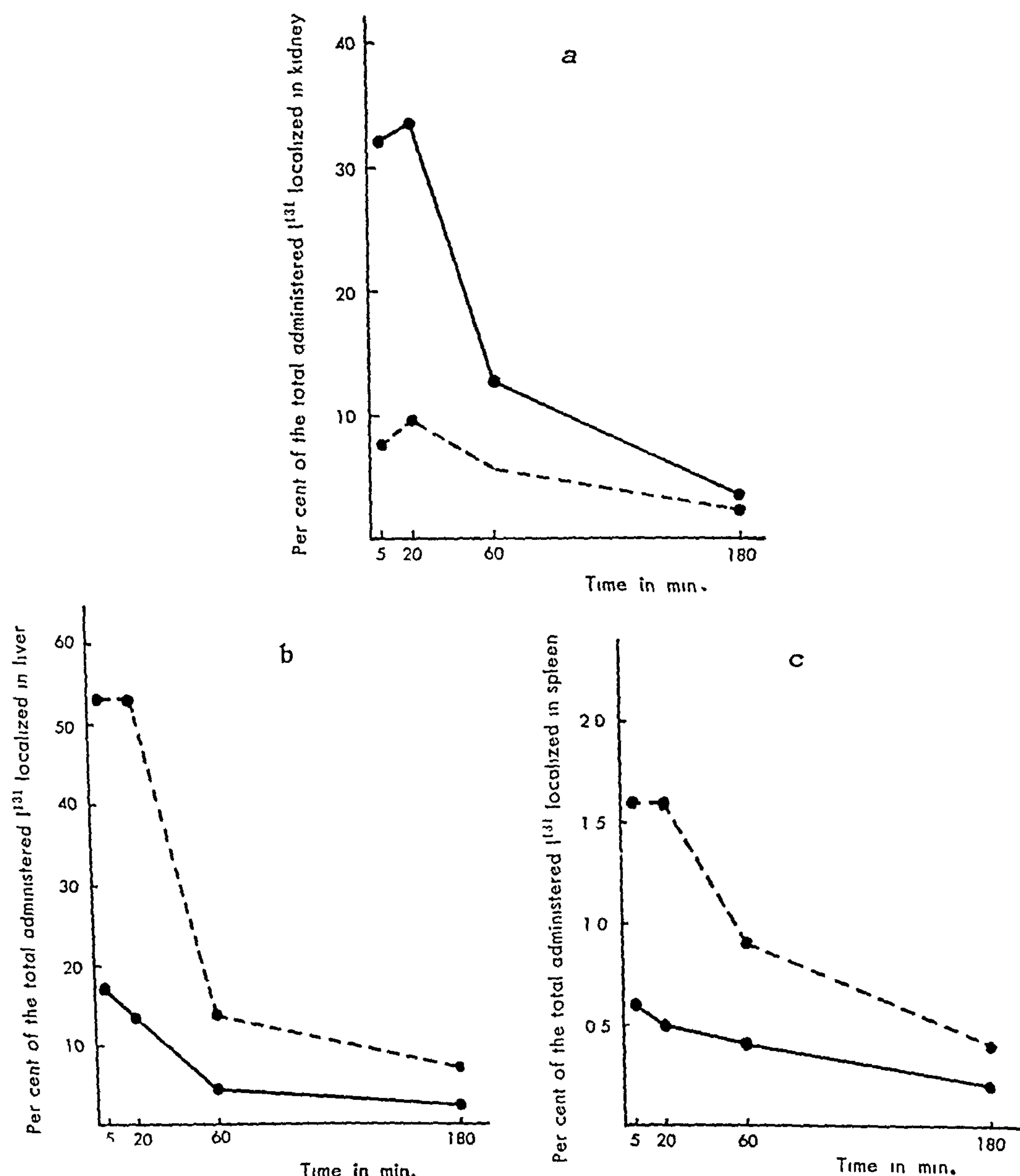


FIGURE 42 Localization of radioactivity in (*a*) kidney, (*b*) liver, and (*c*) spleen after administration of I^{131}-labeled parotin, - -, and I^{131}-labeled saliva parotin A, —— (Reproduced with permission from *Endocrinologia Japonica*.[76])

44 and 45). From the sedimentation constant $s_{20,w}$ and diffusion constant $D_{20,w}$ of saliva parotin A purified only with ethanol, the molecular weight was calculated to be 16,000 (TABLE 17).[78]

Chemical properties. Paper chromatography of the acid and alkaline hydrolyzates of purified saliva parotin A revealed the presence of threonine,

which was not detected in saliva parotin A, and fourteen or fifteen other amino acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine (and/or isoleucine), lysine, phenylalanine, proline, serine, tryptophan,

TABLE 14
LOCALIZATION OF RADIOACTIVITY AFTER INJECTION OF I^{131}-LABELED SALIVA PAROTIN A IN RATS*

Time (min)	Body weight (gm)	Per cent of total administered I^{131} localized in each organ†					
		Blood	Kidney		Liver	Spleen	Thyroid
			Cortex	Medulla			
5	133	22.7	25.9	0.2	17.4	0.3	0.04
	151	24.4	32.9	0.2	16.3	0.7	0.03
	142	22.4	29.4	0.2	18.1	0.8	0.04
	140	21.0	39.9	0.3	16.6	0.6	—
20	146	9.6	25.5	0.3	10.4	0.6	0.2
	158	10.3	36.4	0.3	13.7	0.3	0.1
	140	8.3	33.1	0.2	13.2	0.3	0.3
	146	9.8	39.1	0.2	16.7	0.6	0.2
60	139	5.6	13.6	0.1	7.0	0.5	0.9
	148	6.8	13.6	0.1	2.3	0.3	0.6
	144	8.4	12.0	0.1	3.8	0.3	2.3
	135	7.2	12.0	0.1	5.3	0.3	1.2
180	146	4.6	4.3	0.05	3.3	0.2	3.3
	163	4.2	3.7	0.04	2.4	0.1	4.1
	134	4.2	3.1	0.02	2.2	0.2	4.1
	144	4.6	3.2	0.05	1.7	0.2	3.6

* Reproduced by permission from *Endocrinologia Japonica* [14]
† One-tenth mg I^{131}-labeled saliva parotin A was injected intravenously in each rat.

TABLE 15
FRACTIONATION OF SALIVA PAROTIN A WITH ETHANOL

Fraction (% Solubility)	Yield (%)	No. of rabbits	Dose (μg/kg)	Mean rate of calcium decrease (%)	Leukocyte activity
Original material		3	20	11.27	+
A (33)	8.0	3	10	20.54	+
B (60)	8.7	3	10	17.26	+
C (60)	48.0	3	20	8.94	−

tyrosine, and valine (FIGURE 46). The contents of these amino acids were determined by microbioassay of the acid hydrolyzate of purified saliva parotin A (TABLE 18)

Purified saliva parotin A is most stable in acid solution, retaining its activity in 0.2 N hydrochloric acid, but not in 1.0 N hydrochloric acid after heating at 100° C. for 30 min. When purified saliva parotin A was left in 5.0 N hy-

TABLE 16

FRACTIONATION OF SALIVA A BY ULTRACENTRIFUGATION*

Experiment No. 1

Fraction	Yield mg (%)	Dose (μg /kg)	Animals	Mean calcium decrease (%)
Material: 417 mg. 1% solution		10	3	8 76
		20	3	7 20
		100	5	15 29
4 3 ml 1	213 (51 79)	50	3	5.39
1 2 ml 2	101 (24 22)	5	3	8.43
		50	3	11.91
0 5 ml. 3	92 (22.06)	1	3	7 14
		2	3	10.27
		5	3	12 79
		50	3	17 84
4 (Precip)	9 5 (2 20)	1	3	7 49
		5	4	8 68
		10	3	10 40
		50	2	14.06

Experiment No 2

Fraction	Yield mg. (%)	Dose (μg /kg)	Animals	Mean calcium decrease (%)	Leukocyte activity
Exp. No. 1, Frac 1, 464 mg. 1% solution					
3 2 ml 1-A	135 (29.10)	20	3	5.28	—
		50	3	5 02	—
		300	5	4 76	—
2 0 ml 1-B	168 (36.21)	—	—	—	—
0 8 ml 1-C	134 (28.10)	10	3	2 28	—
		50	5	6.95	—
Exp. No 1, Frac. 3, 232 mg 1% solution					
3 2 ml 3-A	54 (23 27)	10	3	8 45	—
		20	3	5.56	—
2 0 ml. 3-B	71 (30 60)	10	3	7 87	—
0 8 ml. 3-C	91 (39.22)	0 5	3	4 73	+
		1	3	10.50	+
		5	3	12 24	+
		10	3	13.02	+
3-D (Precip)	17 (7 32)	0 5	3	7.33	—
		1	3	8 59	+
		5	3	6 05	+
		20	3	11 02	+

Key: +, increase in activity; –, decrease in activity.
* Ultracentrifuged at 54,000 rpm for 5 hr. (Spinco Model E).

Saliva parotin A

Dissolved in distilled water up to concentration of 1 per cent
Equal volume of ethanol added, with stirring and cooling, mixture allowed to stand overnight, then centrifuged

Precipitate (Yield 15 to 20 per cent) — (Supernatant discarded)

Dissolved in water, dialyzed against distilled water, and filtered.

Filtrate — Precipitate (discarded)

Adjusted to 1 0 per cent concentration; poured into 6 5-ml. centrifuging tube, centrifuged at 56,000 rpm for 4 to 5 hours

Lower layer (1 5 ml) — Upper layer (discarded)

Lyophilized

Purified saliva parotin A (yield. ca 10 per cent)

FIGURE 43 Flow sheet for purification of saliva parotin A by fractionation with ethanol and ultracentrifugation.

drochloric acid for 24 hours at 38° C., only the calcium activity was lost (TABLE 19 and FIGURE 47). When it was heated in aqueous or physiological saline solution for 30 min. at 100° C., the calcium activity was lost and the leukocyte activity was weakened (TABLE 19 and FIGURE 48). Purified saliva parotin A was least stable in basic solution. When it was incubated in 0.1 *N* sodium

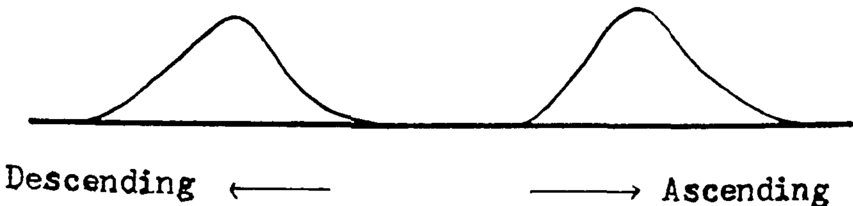


FIGURE 44 Electrophoretic pattern of purified saliva parotin A, 1.0 per cent solution in veronal buffer, *p*H 8.6 (μ = 0.3). Electrophoresis was carried out at 7 mAmp., 50 v., and 3° C. for 40 min. Since saliva parotin A has an extremely weak electric charge and hence the results of electrophoresis are indistinct when the ionic strength of the solution is small, sodium chloride was added to increase the ionic strength to 0.3 per cent.



FIGURE 45 Spinco Model E ultracentrifuge patterns of purified saliva parotin A, 1.0 per cent in 1/15 *M* phosphate buffer solution, *p*H 8.55, 59, 780 rpm. Right to left: patterns at 104, 136, 168, and 200 min.

TABLE 17
SEDIMENTATION AND DIFFUSION CONSTANTS AND MOLECULAR WEIGHT OF SALIVA PAROTIN A

Sedimentation constant $s_{20,w}$	1.51×10^{-13} (cm./sec.)(dyne/gm.) (cm./sec.)(dyne/gm.)
Diffusion constant $D_{20,w}$	7.41×10^{-7} (cm.²/sec.)
Partial specific volume V	0.688
Molecular weight	16,000
Frictional ratio f/f_0	1.76

hydroxide for 2 hours at 38° C., both activities were completely destroyed (TABLE 19 and FIGURE 49).

Although the activities of purified saliva parotin A were not affected by treatment with proteases such as pepsin, trypsin, and chymotrypsin (TABLE 20 and FIGURE 50), it was shown by paper chromatography that purified saliva parotin A was decomposed by proteases into smaller molecules that did not diffuse through a cellophane membrane (FIGURE 51). When purified saliva parotin A was treated in 0.1 *N* sodium hydroxide for 2 hours at 38° C., degradation was clearly evident. After treatment in 5.0 *N* hydrochloric acid for 5 hours at 38° C., purified saliva parotin A was found to be slightly decom-

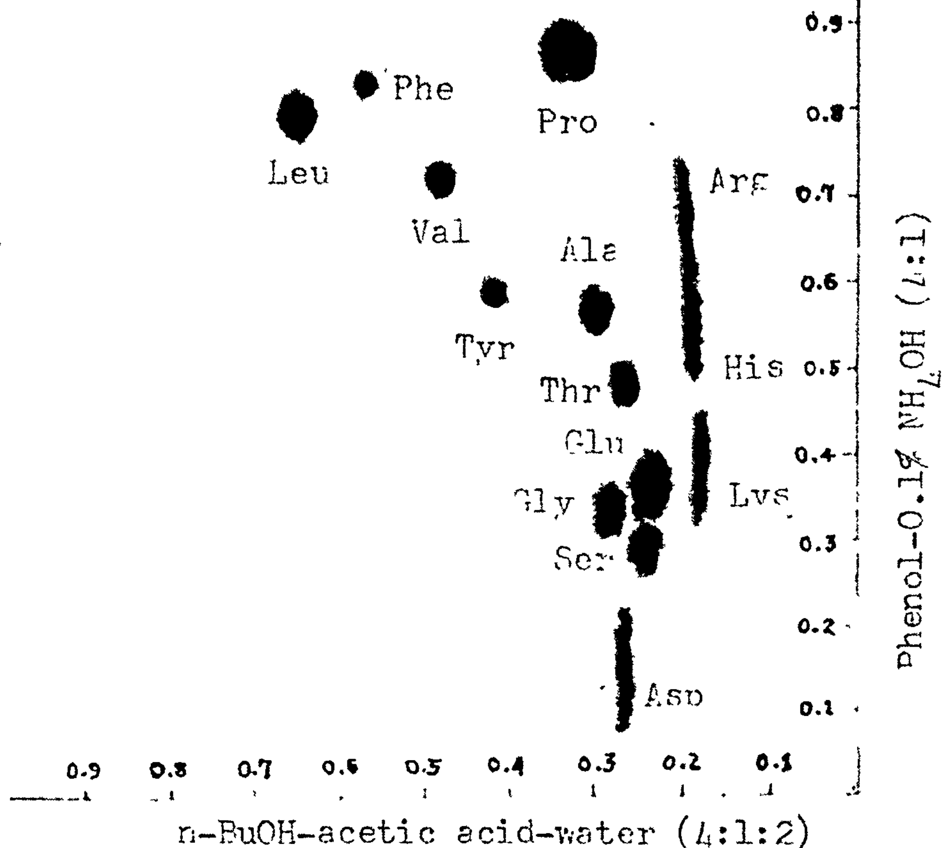


FIGURE 46 Paper partition chromatogram of the HCl-hydrolyzate of purified saliva parotin A.

TABLE 18
AMINO ACID COMPOSITION OF PURIFIED SALIVA PAROTIN A BY MICROBIOASSAY

Amino acid	Amino acid (%)	Amino acid residue (%)	Amino N / Protein N (%)	Microorganisms† used in assay
Alanine	1 00	0 80	1.08	Cit
Arginine	7 14	6 40	15 81	M
Aspartic acid	12 70	10.98	9 19	M
Cystine*	0 60	0 55	0.48	Cit
Glutamic acid	22 25	19.50	15 58	S
Glycine	11 13	8 45	14 29	M
Histidine	2 47	2 18	4 60	Cit
Isoleucine	1 90	1 64	1 39	M
Leucine	3 48	3 00	2.55	M
Lysine	4 88	4.28	6 43	M
Methionine*	0 28	0 25	0 15	M
Phenylalanine	2 08	1 85	1.12	M
Proline	13 72	11 57	11 49	Cit
Serine*	7 98	6 61	7 33	M
Threonine	14 84	12 59	12 22	S
Tryptophan	0 09	0 08	0 06	M
Tyrosine*	1.42	1.28	0 75	M
Valine	3 15	2 66	2.59	M
Total	111 11	94 68	106 25	

* Hydrolyzed with 3 *N* HCl at 120° C for 2 hr Others were hydrolyzed with 3 *N* HCl at 120° C. for 8 hr.

† Cit, *Leuconostoc citrovorum* (ATCC 8081), M, *Leukonostoc mesenteroides* (P-60 ATCC 8042); *Streptococcus faecalis* (R ATCC 8043).

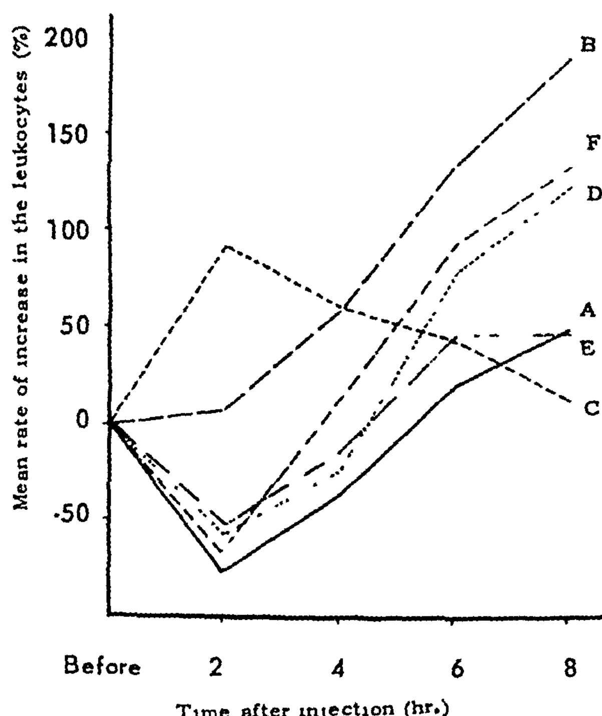


FIGURE 47. Influence of acid and heat treatment on leukocyte activity of purified saliva parotin A. Curve A: 0.1 *N* HCl, 38° C, 24 hours, Curve B 0.2 *N* HCl, 100° C, 30 min; curve C: 1.0 *N* HCl, 100° C., 30 min.; curve D 5.0 *N* HCl, 38° C, 5 hours; curve E: 5.0 *N* HCl, 38° C, 24 hours; curve F. 0.1 *N* CH_3COOH, 60° C., 30 hours Dosage: 10 μg/kg intravenous injection

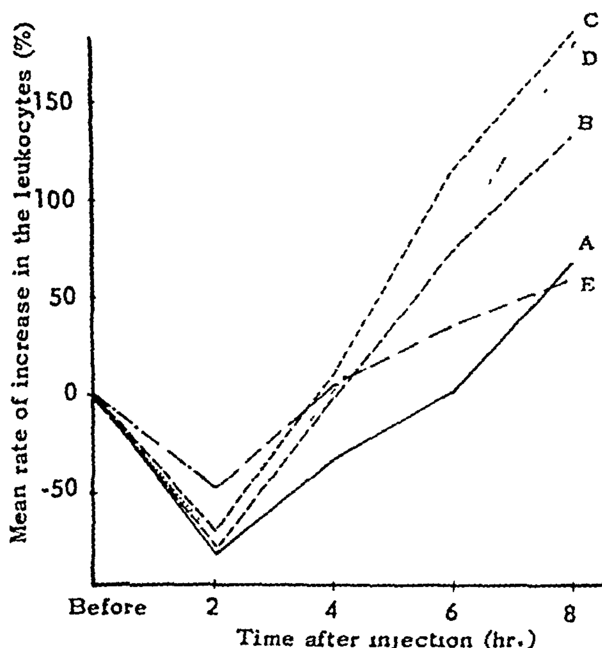


FIGURE 48. Influence of heat treatment on the leukocyte activity of purified saliva parotin A in aqueous or physiological saline solution (mean value for 5 rabbits) Curve A: 38° C., 52 hours, curve B: 23 to 38° C., 5 days; curve C 60° C, 30 min; curve D: 60° C, 1 hour; curve E: 100° C, 30 min. Dosage: 10 μg./kg intravenous injection

TABLE 19

EFFECT OF ACID AND ALKALINE TREATMENT ON ACTIVITIES OF SALIVA PAROTIN A

Solution	Temperature (°C)	Length of treatment (hr)	Percentage serum calcium decrease* Mean ± S E.
Acid			
0 1 *N* HCl (*p*H 1.2)	38	24	10 90 ± 1.29
0 2 *N* HCl	100	0.5	12.88 ± 1 63
1 0 *N* HCl	100	0 5	6 69 ± 0.85
5.0 *N* HCl	38	5	14.30 ± 2.53
5 0 *N* HCl	38	24	7 54 ± 3.07
0 1 *N* CH_3COOH	60	3	13 54 ± 0.53
Neutral	38	52	12 31 ± 2 01
aqueous solution or	23–28	120	13 46 ± 1 96
0.85% NaCl	60	0.5	12.53 ± 1.25
	60	1	12 85 ± 0 87
	100	0 5	6 01 ± 1 24
Alkaline			
0 1 *N* Na_2CO_3 (*p*H 11 2)	17–23	17	10 87 ± 1.45
0.1 *N* Na_2CO_3	17–23	72	8.67 ± 0 74
0 1 *N* NaOH	38	2	4 90 ± 2 24
0 1 *N* Borate buffer (*p*H 8 8)	38	3†	13.40 ± 1.58
Control			
0 85% NaCl		No treatment	14 39 ± 1.61

* Dose of saliva parotin A, 10 μg /kg intravenous injection; mean value of 5 rabbits.
† Dialyzed. Other acid and alkaline solutions were neutralized

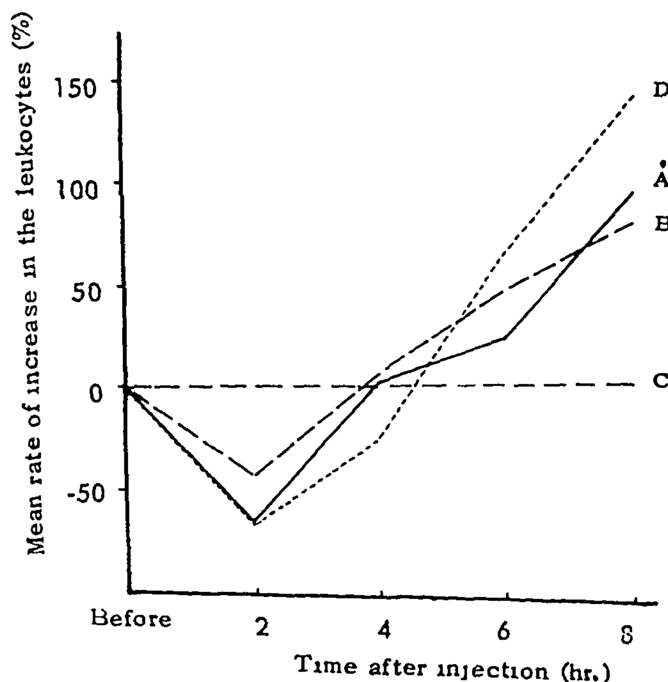


FIGURE 49 Influence of basic treatment on the leukocyte activity of purified saliva parotin A (mean value for 5 rabbits). Curve A 0.1 *M* Na_2CO_3, 17 to 23° C, 17 hours, curve B: 0 1 *M* Na_2CO_3, 17 to 23° C, 72 hours; curve C 0.1 *M* NaOH, 38° C., 2 hours; curve D. borate buffer, *p*H 8 8, 38° C, 3 hours Dosage 10 μg /kg intravenous injection.

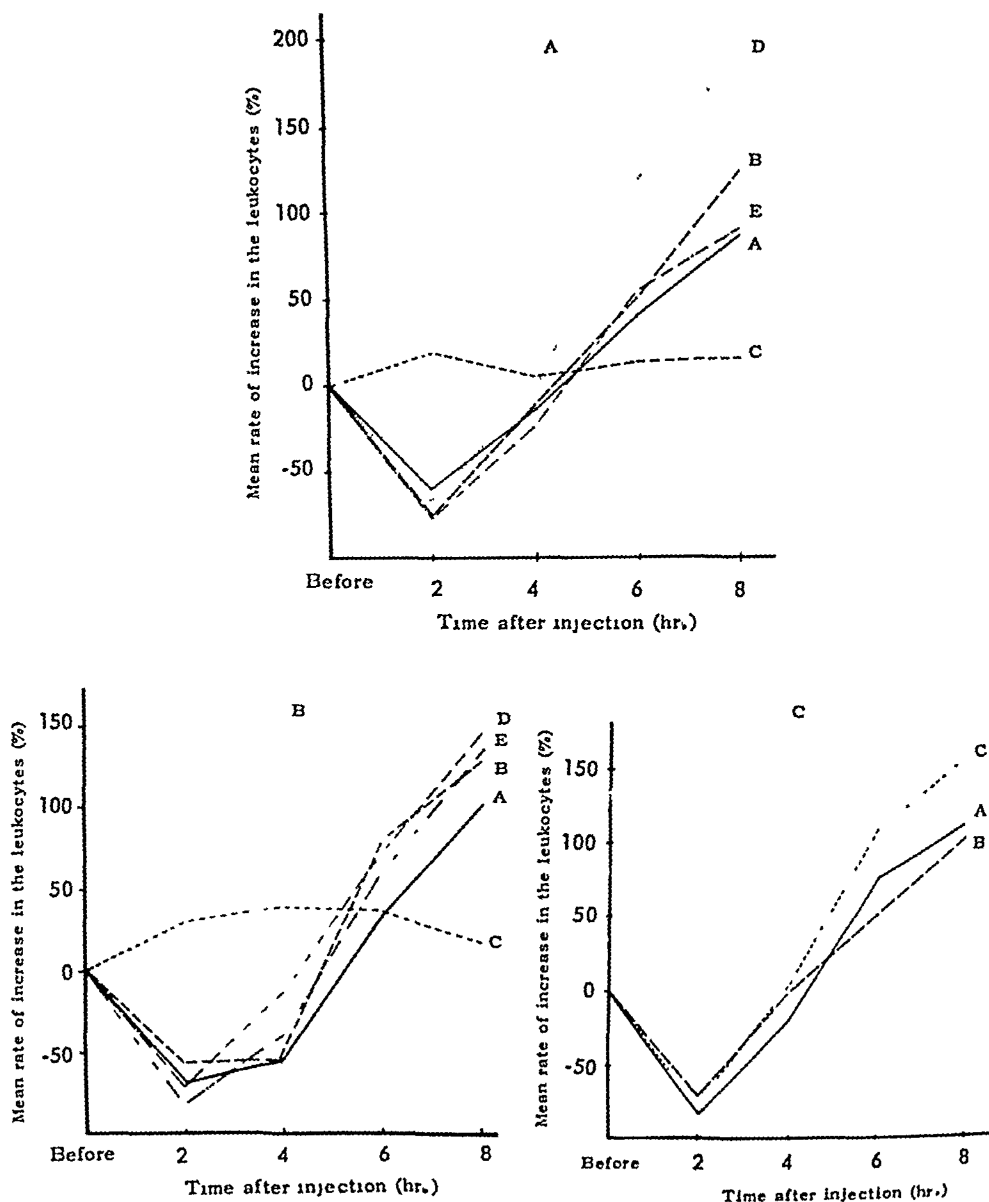


FIGURE 50 (*a*) Influence of pepsin on the leukocyte activity of purified saliva parotin A (mean value for 5 rabbits) Curve A *p*H 2, 38° C., 2 hours (SPA:pepsin 1:1); curve B: *p*H 2, 38° C, 24 hours (SPA:pepsin 1:1), curve C control (10 μg pepsin); curve D: *p*H 2, 38° C, 24 hours (SPA:pepsin 50:1), curve E· dialyzate of material treated as in D. Dosage of saliva parotin A: 10 μg /kg intravenous injection (*b*) Influence of trypsin on the leukocyte activity of purified saliva parotin A (mean value for 5 rabbits) Curve A· *p*H 7 phosphate buffer, 38° C, 2 hours (SPA:trypsin 1:1), curve B *p*H 7 phosphate buffer, 38° C, 24 hours (SPA: trypsin 1:1), curve C control (10 μg trypsin), curve D *p*H 7.5 phosphate buffer, 38° C, 24 hours (SPA:trypsin 50.1); curve E dialyzate of material treated as in D. Dosage of saliva parotin A· 10 μg./kg. intravenous injection (*c*) Influence of chymotrypsin on the leukocyte activity of saliva parotin A (mean value for 5 rabbits) Curve A: *p*H 8 phosphate buffer, 38° C., 2 hours (SPA:chymotrypsin 1:1), curve B. *p*H 8 phosphate buffer, 38° C, 24 hours (SPA:chymotrypsin 1:1); curve C· dialyzate of material treated as in B. Dosage of saliva parotin A: 10 μg./kg. intravenous injection

posed, though its activity was not affected. The biological activities of purified saliva parotin A were destroyed completely by formaldehyde, but not by nitrous acid (TABLE 21 and FIGURE 52).

Treatments with iodine did not influence the activities of purified saliva parotin A (TABLE 21 and FIGURE 53). Furthermore, experiments with radioactive iodine confirmed the supposition that biological activities of purified saliva parotin A would be retained, while tyrosine residues would all be iodinated (TABLE 22 and FIGURE 54) Treatment with thioglycollic acid or cysteine did not modify the activities of purified saliva parotin A (TABLE 21 and FIGURE 55). In contrast to the case of parotin and S-parotin, urea and guanidine hydrochloride did not alter the activities of purified saliva parotin A (TABLE 23 and FIGURE 56). Inactivation by guanidine carbonate must have been due to the alkalinity of the solution.

TABLE 20

INFLUENCE OF PROTEOLYTIC ENZYMES ON ACTIVITIES OF SALIVA PAROTIN A

Enzyme	Buffer solution and concentration of enzyme	Length of treatment (hr)	Neutralized or dialyzed	Percentage serum calcium decrease* Mean ± S E.
Pepsin	*p*H 2 0 HCl, SPA:pepsin (1:1)†	2	Neut.	14 26 ± 1.96
	*p*H 2 0 HCl, SPA:pepsin (1:1)†	24	Neut	12.84 ± 2.11
	Control (*p*H 2 0 HCl, pepsin)	24	Neut.	9.08 ± 1.21
	*p*H 2 0 HCl, SPA:pepsin (50:1)	24	Neut.	13.02 ± 2.51
	*p*H 2.0 HCl, SPA:pepsin (50:1)	24	Dial.	11 93 ± 1.25
Trypsin	*p*H 7.0 phosphate buffer, SPA:trypsin (1:1)	2		14.68 ± 2.53
	*p*H 7 0 phosphate buffer, SPA:trypsin (1:1)	24		13 34 ± 2 12
	Control (phosphate buffer, trypsin)	24		5.43 ± 0 18
	*p*H 7.5 phosphate buffer, SPA:trypsin (50:1)	24		11 80 ± 1.33
	*p*H 7.5 phosphate buffer, SPA:trypsin (50:1)	24	Dial	12.71 ± 1 96
Chymotrypsin	*p*H 8 0 phosphate buffer, SPA:chymotrypsin (1:1)	2		14 78 ± 2 21
	*p*H 8 0 phosphate buffer, SPA:chymotrypsin (1:1)	24		14.85 ± 1 87
	*p*H 8 0 phosphate buffer, SPA:chymotrypsin (1:1)	24	Dial.	14.86 ± 2 57
Control	0 85% NaCl solution	No treatment		14 39 ± 1.61

* Dose of saliva parotin A, 10 μg /kg intravenous injection; mean value of 5 rabbits. All experiments carried out at 38° C

† SPA, saliva parotin A The figures in parentheses are weight ratios

From the finding that purified saliva parotin A, in contrast to parotin and S-parotin, was inactivated only by formaldehyde, it can be deduced that the reactive groups responsible for the biological activities of saliva parotin A might be different from those of the latter proteins.

Biological activities. The biological potencies of purified saliva parotin A

are very high. The minimum effective doses per kg. body weight by intravenous injection in rabbits were found to be as follows: 5 to 10 μg. for calcium activity (FIGURE 57), 0.25 μg for leukocyte activity (FIGURE 58), and 2 to 5 μg. for dentine-calcification activity.[78]

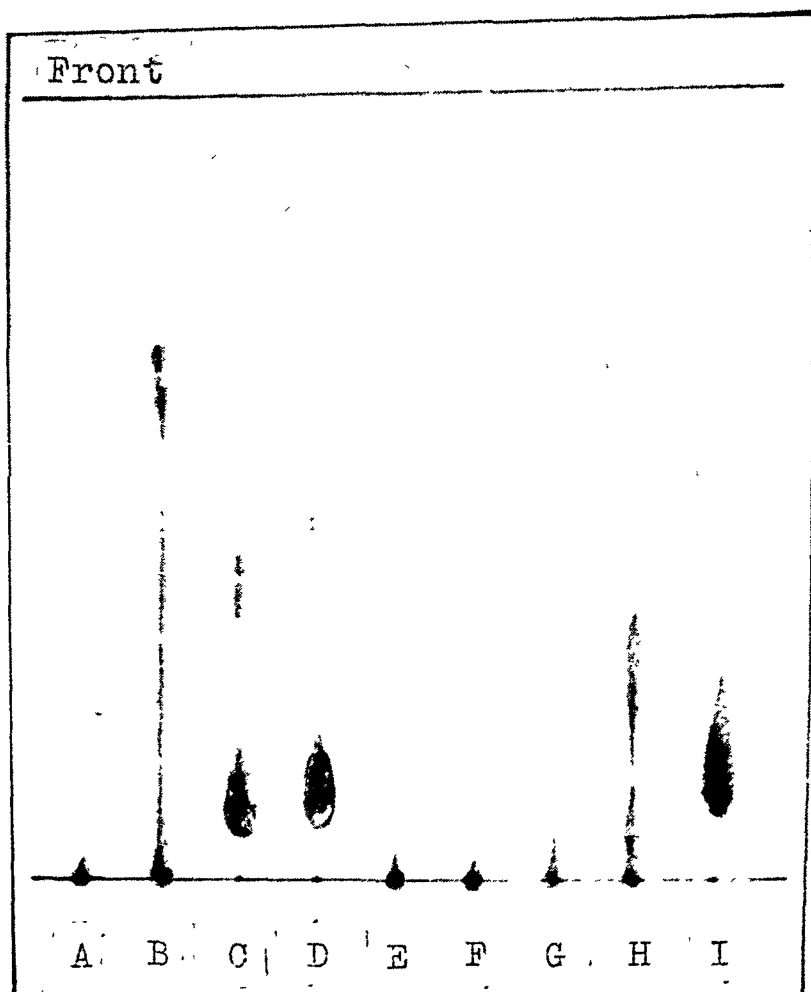


FIGURE 51. Paper partition chromatogram of purified saliva-parotin-A solution treated with HCl, NaOH, and proteases. A: control (untreated), B treated with pepsin, C: treated with trypsin; D: treated with chymotrypsin; E. 0 1 *N* HCl, 38° C, 24 hours; F: 0 2 *N* HCl, 100° C, 30 min; G 1 *N* HCl, 100° C, 30 min; H: 5 *N* HCl, 38° C., 30 min; I 0 1 *N* NaOH, 38° C, 2 hours Developing solvent. *n*-butanol-acetic acid-water (4:1:5).

Using the tissue culture method, my associates and I demonstrated the promotive effect of purified saliva parotin A on the development of bone; it was identical with that of parotin except that it could be observed only at much lower concentrations (Ito, Endo, and Enomoto, unpublished).

Nine-day chick embryo femora were cultivated for 6 days according to the roller-tube method described above, with the exception that in these experiments 13-day chick embryo extract was used exclusively throughout the culture. The length of the bones was measured every day with the ocular

TABLE 21

INFLUENCES OF CHEMICAL REAGENTS ON ACTIVITIES OF SALIVA PAROTIN A

Reagents	Buffer solution and concentration of reagents	Temperature (°C)	Length of treatment (hr)	Length of dialysis (hr)	Percentage serum calcium decrease* Mean ± S E
Nitrous acid	pH 4 0 acetate buffer, 1 0 M	0	1	24	17.55 ± 2 95
	pH 4 0 acetate buffer, 1 0 M	0	24	24	16.05 ± 1 08
	pH 4.0 acetate buffer, 3 0 M	38	1	0	13 28 ± 1.38
	Control (acetate buffer, 3 0 M, $NaNO_2$)	38	1	0	5.90 ± 0.94
Formaldehyde	pH 8 0 phosphate buffer, 15% solution	38	0 5	48	5.70 ± 0.78
Iodine	pH 7 0 phosphate buffer, 0.66 M	15	1	24	17.20 ± 2.51
	pH 7 0 phosphate buffer, 0 66 M	30	24	24	13 29 ± 1.28
Cysteine	pH 7 4 phosphate buffer, SPA:cysteine (1:50)†	13–17	72	24	12.62 ± 2 16
Thioglycollic acid	pH 7 4 phosphate buffer, 1 0 M	12–19	96	48	12 84 ± 1.21
	pH 7.4 phosphate buffer, 2 0 M	13–17	72	48	13 95 ± 2.25
	Control (phosphate buffer, 2 0 M, thioglyc)	13–17	72	48	4.97 ± 0.32
Control	0 85% NaCl solution		No treatment		14.39 ± 1 61

* Dose of saliva parotin A, 10 μg /kg intravenous injection; mean value of 5 rabbits
† SPA, saliva parotin A.

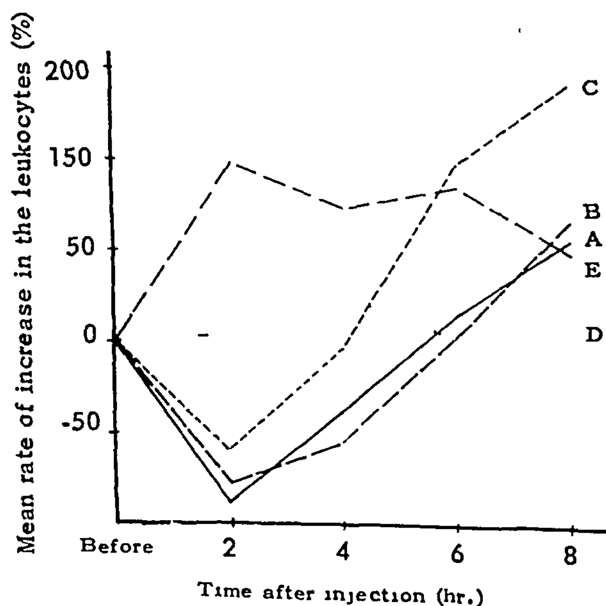


FIGURE 52 Influences of nitrous acid and formaldehyde on the leukocyte activity of saliva parotin A (mean value for 5 rabbits) Curve A 1 M nitrous acid, 0° C , 1 hour; curve B 1 M nitrous acid, 0° C , 24 hours; curve C 3 M nitrous acid, 38° C , 1 hour; curve D control (3 M nitrous acid solution); curve E 15 per cent formaldehyde solution, 38° C., 30 min Dosage of saliva parotin A· 10 μg /kg intravenous injection.

micrometer of a microscope. After the dry weight of each bone had been obtained, the amount of calcification was estimated from determinations of phosphorus extracted at 50° C. for 3 hours with 3 ml. of 5 per cent trichloroacetic acid. The degree of formation of cartilage matrix was determined from estimations of hydroxyproline in the acid hydrolyzate of the extracted bones.

FIGURE 59*b* and *c* show the stimulative effect of purified saliva parotin

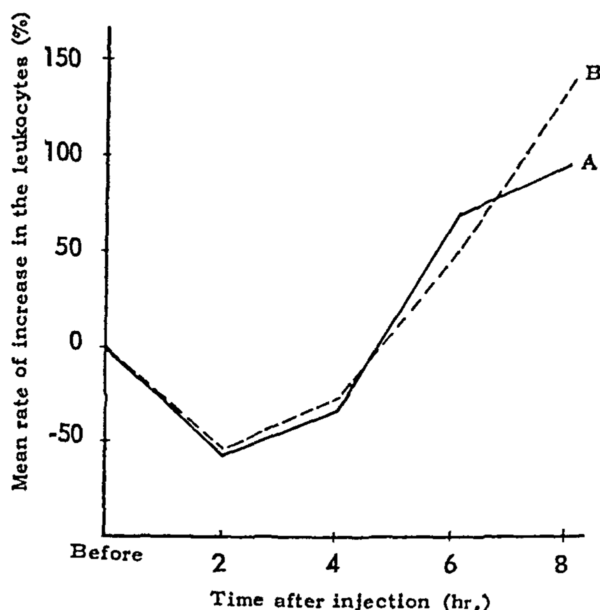


FIGURE 53 Influence of iodine on the leukocyte activity of saliva parotin A (mean value for 5 rabbits). Curve A: 0 66 *M*, I (KI), 15° C , 1 hour, curve B 0 66 *M*, I (KI), 30° C., 24 hours. Dosage of saliva parotin A 10 μg /kg intravenous injection.

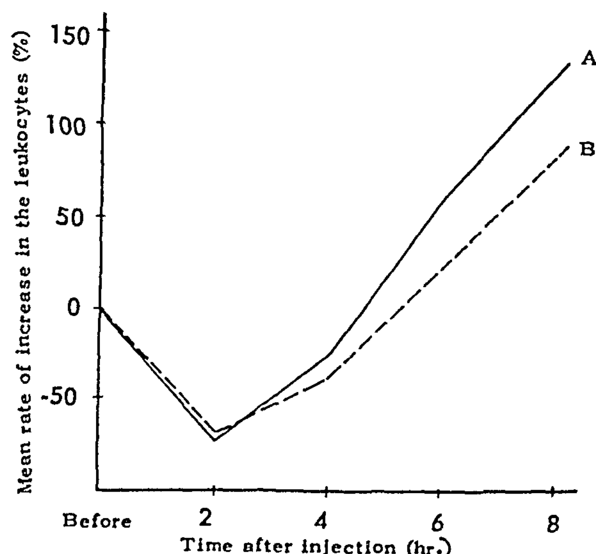


FIGURE 54 Leukocytic activity of I^{131}-labeled saliva parotin A (mean value for 5 rabbits). Curve A: treated with 0 002 *N* KI (labeled with I^{131}); curve B treated with 0 025 *N* KI (labled with I^{131}). Dosage of I^{131}-labeled saliva parotin A: 10 μg./kg. intravenous injection.

A on the longitudinal growth of the bones at concentrations of 0.05 and 0.1 γ/ml. of the medium. The effect reversed, however, at concentrations higher than 1.0 γ/ml. (FIGURES 59*e* and 59*f*), and the inhibitory effect at 10 γ/ml.

TABLE 22
CALCIUM ACTIVITY OF I^{131}-LABELED SALIVA PAROTIN A

Experiment No.	Treatment	Number of iodine atoms bound per mg. of SPA*	Percentage serum calcium decrease† Mean ± S E.
I	0.002 *N* KI^{131} iodination (*p*H 8 8, borate buffer, 2.5 hours)	6.8×10^{-8}	12.38 ± 1.33
II	0.012 *N* KI^{131} iodination (*p*H 8.8, borate buffer, 2.5 hours)	4.3×10^{-7}	13.55 ± 2 47
Control	Untreated	—	14.39 ± 1.62

* Saliva parotin A
† Dose of saliva parotin A, 10 μg./kg intravenous injection; mean value of 5 rabbits.

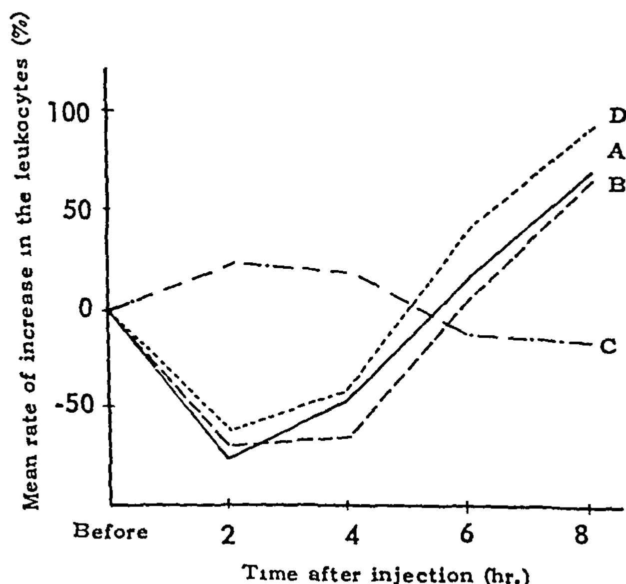


FIGURE 55. Influences of thioglycollic acid and cysteine on the leukocyte activity of saliva parotin A (mean value for 5 rabbits) Curve A. 1 *M* thioglycollic acid, 12 to 19° C, 4 days; curve B 2 *M* thioglycollic acid, 13 to 17° C, 3 days; curve C· control (2 *M* thioglycollic acid solution), curve D: 50 vol cysteine, 13 to 17° C, 3 days Dosage of saliva parotin A 10 μg /kg intravenous injection.

was significant ($p < 0.05$). Although the stimulative effect at lower concentrations was not statistically significant, it was indicated clearly that the over-all tendency was essentially the same as that of parotin (FIGURE 26).

FIGURE 60 shows the effect of purified saliva parotin A on some aspects of bone development under the above conditions. Each bar represents the mean development, the development of each bone being expressed as a percentage of the development of the control bone from the same embryo.

The most remarkable result was that the amount of phosphorus increased over a wide range of concentration of purified saliva parotin A (0.01 to 1.0 γ/ml.), while the dry weight and amount of hydroxyproline showed no such clear tendencies. At concentrations of 0.01 and 0.5 γ/ml., where no effect on longitudinal growth could be observed, the increase in phosphorus was of high statistical significance; even at a concentration of 1.0 γ/ml. such a tendency persisted despite a statistically significant decrease in dry weight of the

TABLE 23

INFLUENCES OF UREA AND GUANIDINE ON ACTIVITIES OF SALIVA PAROTIN A

Reagents	Buffer solution and concentration of reagents	Temperature (°C)	Length of treatment (hr)	Length of dialysis (hr)	Percentage serum calcium decrease* Mean ± S.E
Urea	*p*H 8 0 phosphate buffer, 2 0 *M*	25–30	24	3	12.73 ± 2.49
	*p*H 8 0 phosphate buffer, 5.0 *M*	25–30	24	3	13.03 ± 1 68
Guanidine	*p*H 8.0 phosphate buffer, guanidine-HCl, 5 0 *M*	14–24	192	48	16.93 ± 2 28
	*p*H 8.0 phosphate buffer, guanidine-H_2CO_3 (alkali), 5 0 *M*	38	42	48	4.09 ± 1.05
Control	0 85% NaCl solution		No treatment		14.39 ± 1 61

* Dose of saliva parotin A, 10 μg /kg intravenous injection; mean value of 5 rabbits

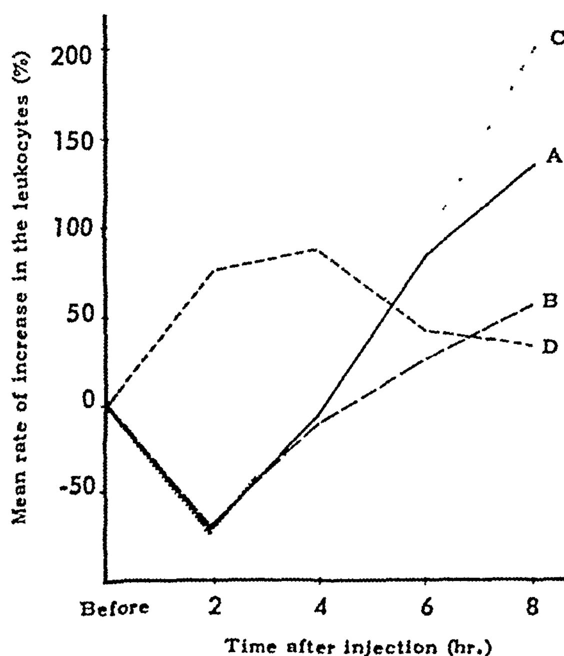


FIGURE 56. Influences of urea and guanidine on the leukocyte activity of purified saliva parotin A (mean value for 5 rabbits) Curve A 2 *M* urea, 25 to 30° C., 24 hours; curve B: 5 *M* urea, 25 to 30° C., 24 hours; curve C. 5 *M* guanidine-HCl, 14 to 24° C, 3 days, curve D 5 *M* guanidine-H_2CO_3, 38° C, 42 hours Dosage of saliva parotin A: 10 μg./kg. intravenous injection

bone corresponding to the marked inhibition of the longitudinal growth (TABLE 24). The phosphorus determined must derive from bone salt deposited as calcium phosphate and thus must indicate the degree of calcification; the amount of hydroxyproline estimated reflects, possibly, the degree of formation of the cartilage matrix.

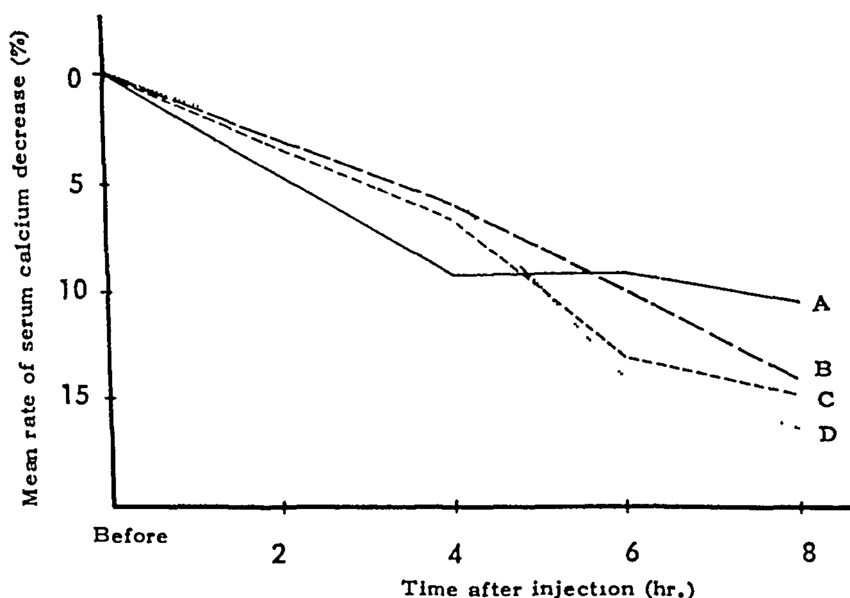


FIGURE 57. Effect of purified saliva parotin A on the rabbit serum calcium level (mean value for 5 normal rabbits) Intravenous injection of saliva parotin A curve A, 2 5 μg./kg , curve B, 5 μg /kg ; curve C, 10 μg /kg ; curve D, 20 μg /kg.

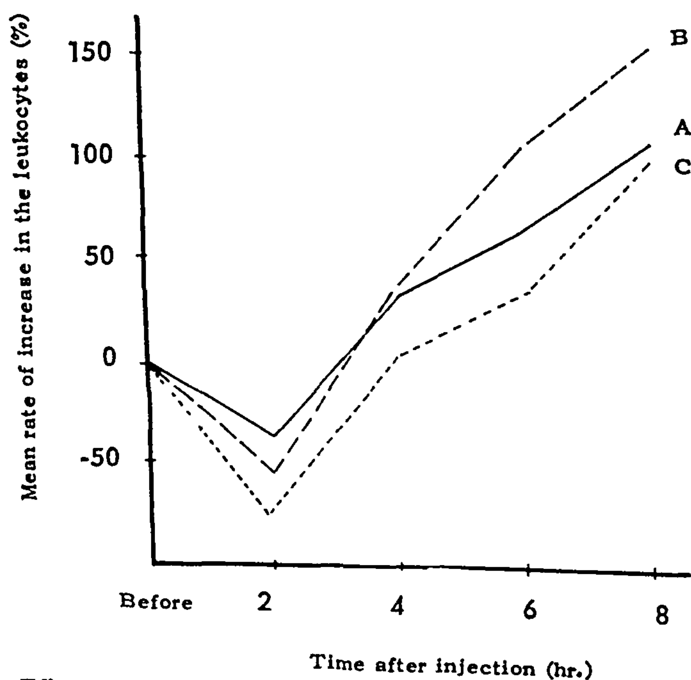


FIGURE 58 Effect of purified saliva parotin A on the number of circulating leukocytes in rabbits (mean value for 5 normal rabbits) Intravenous injections of saliva parotin A. curve A, 0 25 μg /kg , curve B, 0 5 μg /kg , and curve C, 1 μg /kg

From these findings it may be concluded that saliva parotin A has a direct, stimulative effect upon the calcification of bone under certain optimal conditions. Furthermore, because purified saliva parotin A, remarkably more potent than parotin in calcium activity, accelerated the development of bone in tissue culture at an optimal concentration much lower (0.1 γ/ml.) than

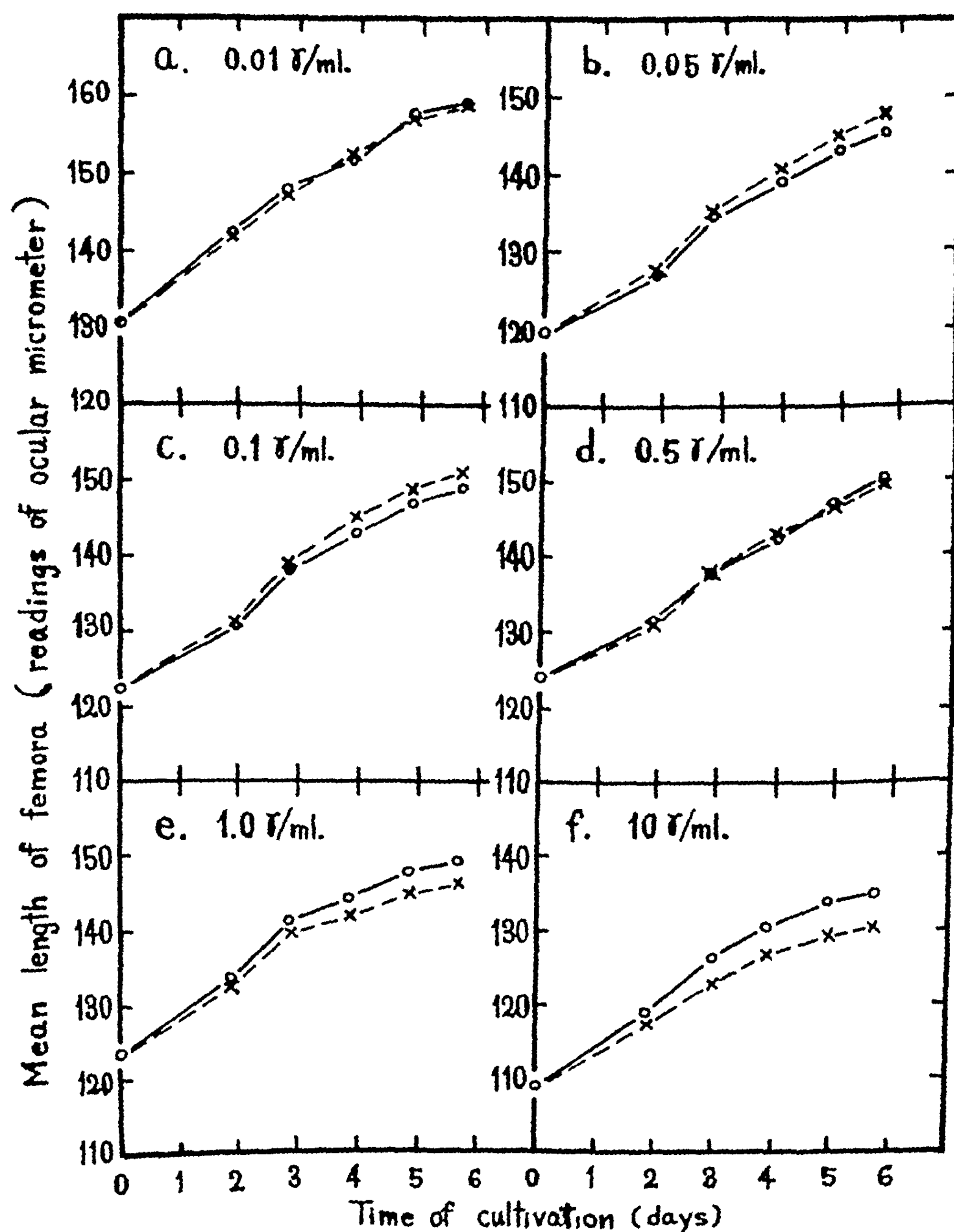


FIGURE 59. Effect of purified saliva parotin A on the longitudinal growth of 9-day chick embryo femora in tissue culture. Mean length for test group treated with saliva; parotin A, X; for control group, O.

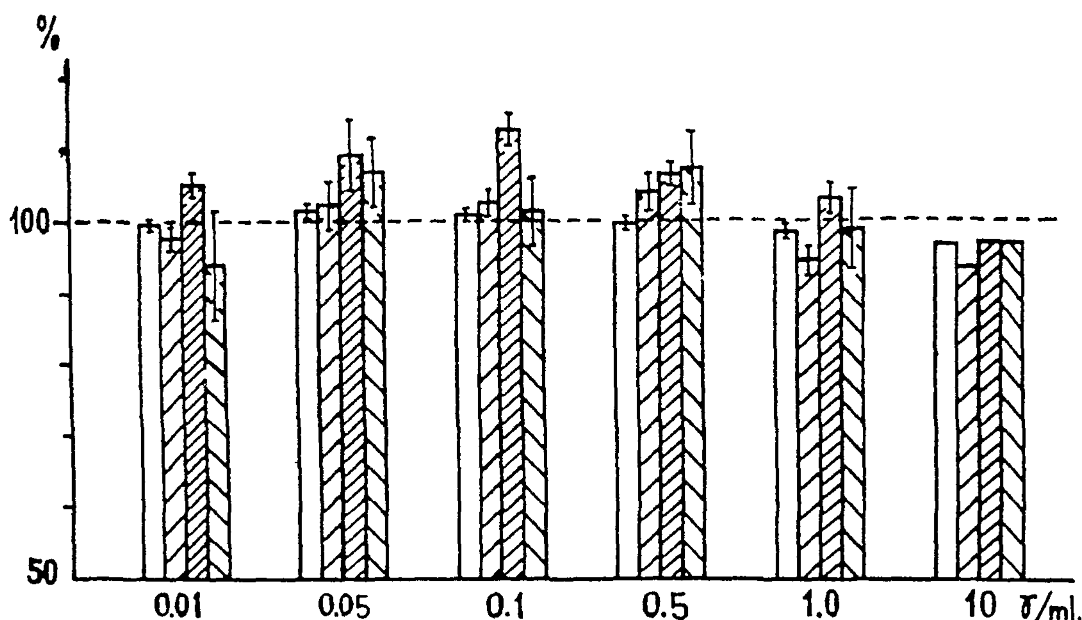


FIGURE 60. Effects of purified saliva parotin A on the chemical development of 9-day chick embryo femora in tissue culture Each bar represents the mean growth of the bone treated with purified saliva parotin A expressed as a percentage of the growth of the control femora from the same embryo The vertical line at the top of each bar represents the total standard error of the mean. The four bars represent, from left to right, length, dry weight, phosphorus, and hydroxyproline.

TABLE 24
EFFECT OF PURIFIED SALIVA PAROTIN A ON THE PHOSPHORUS, HYDROXYPROLINE, AND DRY WEIGHT OF 9-DAY CHICK EMBRYO FEMORA IN TISSUE CULTURE

Concentration of saliva parotin A (γ/ml)	No of pairs	Phosphorus, (hydroxyproline), and [dry weight]* of a femur		Variance of the difference	Level of significance
		Control group	Treated group		
0.01	9	12 6 (13.4) [1084.0]	13.2 (12 1) [1058.1]	0 4411	$p < 0\ 025$
0 1	11	11.9 (12.6) [981 0]	13 4 (12 7) [1005.5]	0 8456	$p < 0\ 001$
0 5	10	13 5 (13 7) [955 3]	14 3 (14 5) [991.2]	0.4579	$p < 0\ 005$
1 0	10	13 8 (13 3) [1043 6]	14.2 (13 2) [989 9]	5453.4	$p < 0\ 05$

* In γ.

that for parotin (150 γ/ml.), an intimate interrelationship is assumed to exist between the activity of these two substances in stimulating development of hard tissues and their calcium activity.

Uroparotin

Assuming that parotin may be excreted into urine after being metabolized into relatively small molecules, my associates and I[79] some years ago isolated a substance with calcium activity from human urine, using benzoic acid adsorption or acetone precipitation. We purified this substance by the method used for bovine parotin. In addition to calcium activity, uroparotin showed leukocyte and dentine-calcification activities, and raised rabbit bone marrow temperature. All activities were much weaker than those in parotin. Uroparotin gave positive results in biuret, xanthoprotein, Millon's, and Adamkiewitz's reactions, but a negative result in a Ninhydrin test

Urine
|adjusted to *p*H 4 5, 2 vol acetone added, centrifuged
Precipitate
|dissolved in 3 vol. water at *p*H 8 0, centrifuged, dialyzed against tap water Dialyzate
| adjusted to *p*H 3 0, centrifuged, washed with acetone, and dried *in vacuo*
Crude uroparotin (FR)
|dissolved in 0.2 *M* NaCl, *p*H 7 0
|Ethanol added, up to 40% concentration at 0° C, centrifuged
Supernatant
|Ethanol added up to 60% concentration at 0° C, centrifuged
Precipitate
|dialyzed against distilled water (ethanol fractionation being repeated for the dialyzate).
|Final precipitate is dialyzed and lyophilized
Ethanol fractionation product (FE)
|FE (500 mg) applied to alumina column 2 5 × 40 cm, eluted with distilled water Effluents added to ethanol, centrifuged, dialyzed, and lyophilized
Alumina column fraction (FC_1)
|FC_1 (150 mg) applied to alumina column 2 0 × 30 cm, eluted with distilled water Effluents are treated as above
Alumina column fraction (FC_2)
|FC_2 (80 mg) applied to alumina column 1 2 × 30 cm, eluted with distilled water Effluents are treated as above
Final alumina column fraction (FC_3)

Figure 61. Isolation of uroparotin from human urine

Isolation[80,81,83]

Recently, M. Yamamoto and I succeeded in isolating relatively powerful uroparotin from human urine as a homogeneous glycoprotein, using an alumina column treatment. Urine was collected from healthy young men during the summer (from June to September) to obtain α-uroparotin and during the winter (from December to March) to obtain β-uroparotin. Uroparotin was isolated as shown in figure 61.

Extraction of crude uroparotin. The urine was brought to *p*H 4.5 with hydrochloric acid, and 2 volumes of acetone were added. After overnight refrigeration, the precipitate was separated by centrifugation, suspended in 3 volumes of water, and extracted with stirring for 3 hours at *p*H 8.0. After centrifugation, the supernatant was dialyzed against tap water for 2 days and

brought to *p*H 3.0 with hydrochloric acid. The precipitate was washed with acetone and ether and dried *in vacuo* (Fraction FR). The smallest dose found to affect calcium activity in rabbits was 2.0 mg./kg. body weight.

Ethanol fractionation. Fraction FR was dissolved in 0.2 *M* sodium chloride, *p*H 7.0, at a concentration of 1.5 per cent. After centrifugation, ethanol was added to the supernatant up to 40 per cent concentration in ice bath, and the precipitate was discarded. To the supernatant, ethanol was added up to 60 per cent concentration in ice bath. After centrifugation, the precipitate was dissolved in a small amount of water and dialyzed against distilled water. This ethanol fractionation was repeated. The final precipitate was dissolved in a small amount of water, dialyzed against distilled water, and lyophilized (Fraction FE).

Alumina column treatment. Fraction FE dissolved in distilled water at a concentration of 5 per cent was adsorbed at the upper end of an alumina

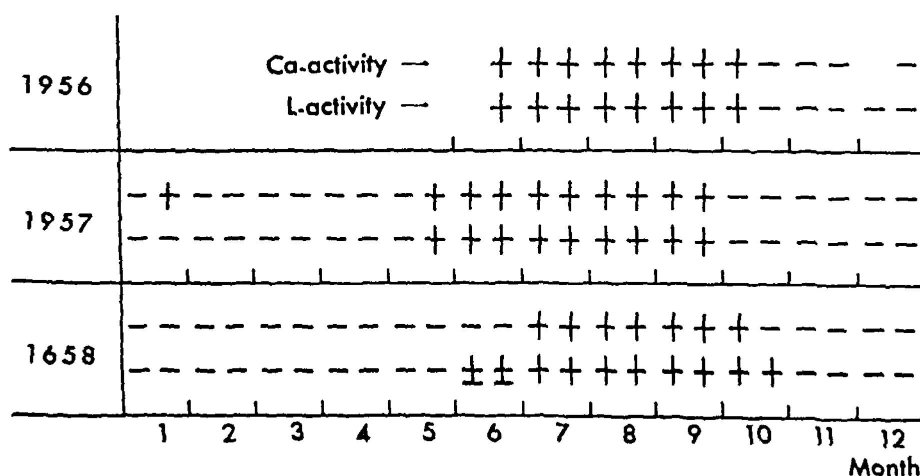


FIGURE 62. Seasonal variation in the activities of uroparotin. The activities of crude uroparotin were estimated with a dosage of 2 0 mg /kg.

column and eluted with distilled water. The eluate was collected in 10-ml. amounts and the optical density for each 10-ml. tube was estimated at 277 mμ. The eluate samples showing maximum adsorption were pooled, sodium chloride was added up to 0.2 *M* concentration, and the mixture was diluted with four volumes of ethanol in ice bath. After centrifugation, the precipitate was dissolved in a small amount of water, dialyzed against distilled water, and lyophilyzed (Fraction FC_1). Fractions FC_2 and FC_3 were obtained by similar alumina column treatments.

Seasonal Variation of Uroparotin Activities[82]

It was found that calcium and leukocyte activities were totally absent in crude uroparotin (Fraction FR) extracted from the urine collected in winter (FIGURE 62 and TABLE 25). For convenience, the active substance extracted from summer urine was termed α-uroparotin and the inactive substance from winter urine, β-uroparotin. Furthermore, as shown in FIGURE 63, no calcium activity could be found in β-uroparotin, regardless of the size of the dose administered.

β-Uroparotin extracted from winter urine and incubated at 37° C. for 24 hours, however, exhibited definite biological activities (TABLE 26). As shown in TABLE 27, crude and purified β-uroparotin were activated through incuba-

TABLE 25
YIELD AND ACTIVITY OF VARIOUS FRACTIONS OBTAINED DURING ISOLATION OF UROPAROTIN

Fraction	Procedure	α-Uroparotin			β-Uroparotin		
		Yield (mg /l urine)	Calcium activity*	Leukocyte activity	Yield (mg /l urine)	Calcium activity	Leukocyte activity
FR	Acetone precipitation at isoelectric point	26.0	2 0	+	20 0	−	−
FE	Ethanol fractionation	14 0	1.0	+	11 0	−	−
FC_1	First alumina treatment	5.0	0.4	+	4.0	−	−
FC_2	Second alumina treatment	2 5	0 2	+	2.0	−	−
FC_3	Final alumina treatment	1 0–1 2	0 1	+	0 8	−	−

* Minimum effective dose, mg /kg. body weight in rabbits
Key: +, activity evident; −, activity not observed

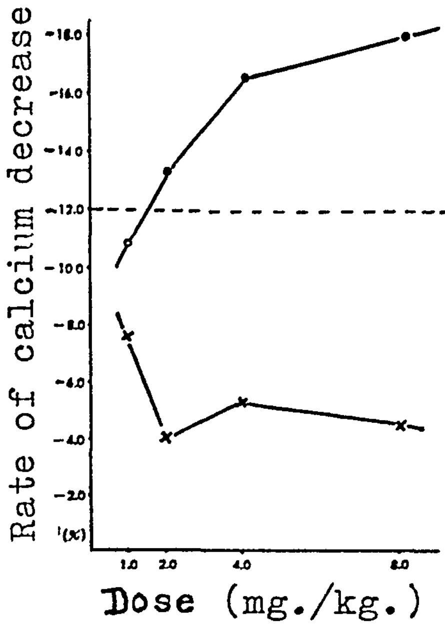


FIGURE 63 Dosage-response curves of crude α- and β-uroparotin. O, crude α-uroparotin; ×, crude β-uroparotin.

tion, the following conditions being found most suitable: *p*H 5.0, 37° C., and 24 to 48 hours. No additional activator was considered to be necessary, since activation occurred even in purified β-uroparotin (Fraction FC_1). The inactivation of β-uroparotin might have occurred in the body, because no activity was observed in the β-uroparotin extracted from fresh winter urine (TABLE 28).

TABLE 26

ACTIVITIES OF CRUDE UROPAROTIN OBTAINED FROM INCUBATED WINTER URINE

Experiment No.	Date	Treatment of urine			
		Incubated (37° C , 24 hr.)		Not incubated	
		Calcium activity	Leukocyte activity	Calcium activity	Leukocyte activity
I	Dec. 1957	+	+	−	−
II	Jan. 1958	±	+	−	−
III	Feb. 1958	+	+	−	−
Dose (mg./kg)		2.0		2.0	

TABLE 27

ACTIVATION OF PURIFIED β-UROPAROTIN BY INCUBATION AT 37° C. UNDER VARIOUS CONDITIONS

Experimental conditions		Activation of fractions			
		Urine*	FR	FE	FC_1
pH (μ = 0 12, 24 hr.)	8.0	−	−	−	−
	6.3	±	±	±	±
	5 0	+	+	+	+
Salt concentration, μ (pH 5.1, 24 hr)	0.65		+	+	±
	0.12		+	+	±
Time, hr. (pH 5.1, μ = 0 12)	12	−	−	−	−
	24	+	+	+	+
	48	±	±	±	±
	72	−	−	−	−
Intact control		−	−	−	−
Dose (mg /kg)		2.0	2.0	1 0	0 25

* Incubated urine was treated to obtain crude uroparotin (FR).

TABLE 28

ACTIVITIES OF β-UROPAROTIN OBTAINED FROM FRESH URINE

Experiment No	Date	Fresh urine		Control urine*	
		Calcium activity	Leukocyte activity	Calcium activity	Leukocyte activity
I	Oct. 29–31	+	−	−	−
II	Nov 5–8	−	−	−	−
III	Dec 25–26	+	+	−	−
IV	Dec. 2–4	−	−	−	−
V	Dec 5–9	−	−	−	−
VI	Dec. 16–18	−	−	−	−
VII	Jan 19–21	−	−	−	−
Dose (mg /kg)		2.0		2 0	

* The control urine was refrigerated for 12 to 24 hours.

Excretion of Uroparotin in Salivary Glandectomized Animals

My associate and I studied the effect of excision of salivary glands on the excretion of uroparotin in rabbits, in which uroparotin activity shows no seasonal variation (Ito and Yamamoto, unpublished). The amount of uroparotin excreted into urine decreased markedly after excision of parotid and/or submaxillary glands. In all the extirpated animals, however, the excretion of uroparotin about 80 weeks after the operation was found to be restored to the normal level, both in yield and activities (FIGURE 64). From these results,

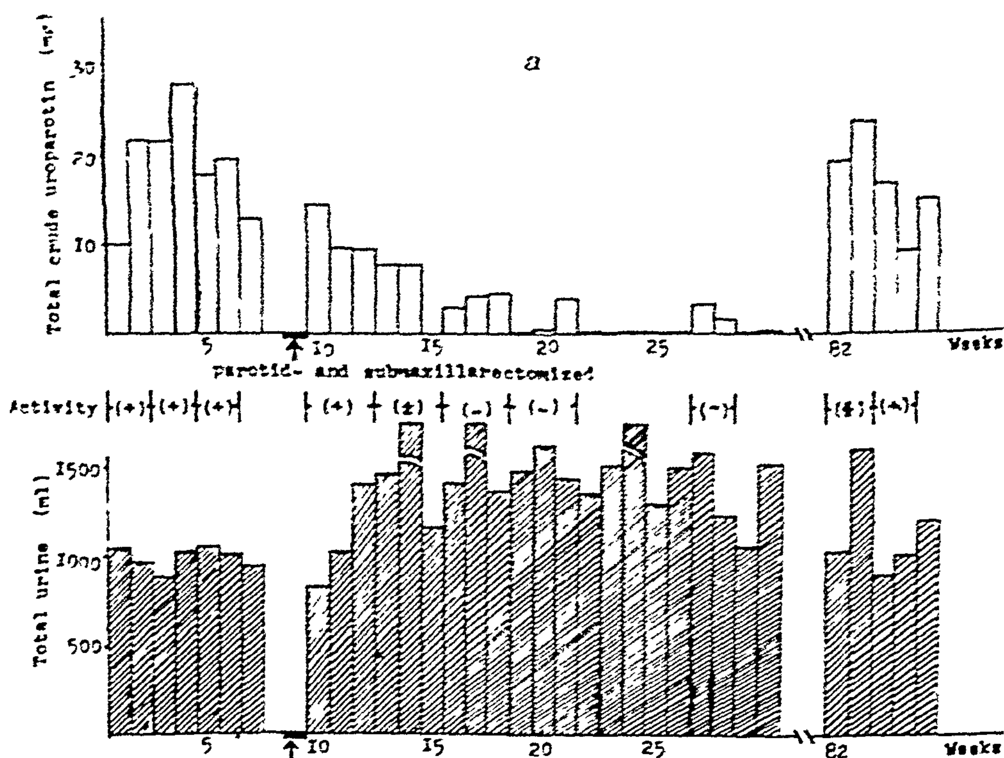


FIGURE 64. (a) Effect of parotidectomy and submaxillarectomy on rabbit uroparotin. Both parotid and submaxillary glands were ectomized in the ninth week. Minimum doses affecting activities: ++, <0.5 mg./kg.; +, 1.0 mg./kg.; ±, 2.0 mg./kg.; −, >4.0 mg./kg.

it was assumed that the function of the salivary glands and the excretion of uroparotin may be closely related.

Chemical Properties[53]

Uroparotin was found to be almost homogeneous, by ultracentrifugal as well as electrophoretic analysis in several types of buffer solutions (FIGURES 65 and 66). The isoelectric point of both α- and β-uroparotin was calculated to be *p*H 2.9, from results of paper electrophoresis in which bovine serum albumin was used as control and dextran for correction (FIGURE 67).

Two-dimensional and circular paper chromatography revealed the following seventeen amino acids in the hydrolyzates of α- and β-uroparotin: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine,

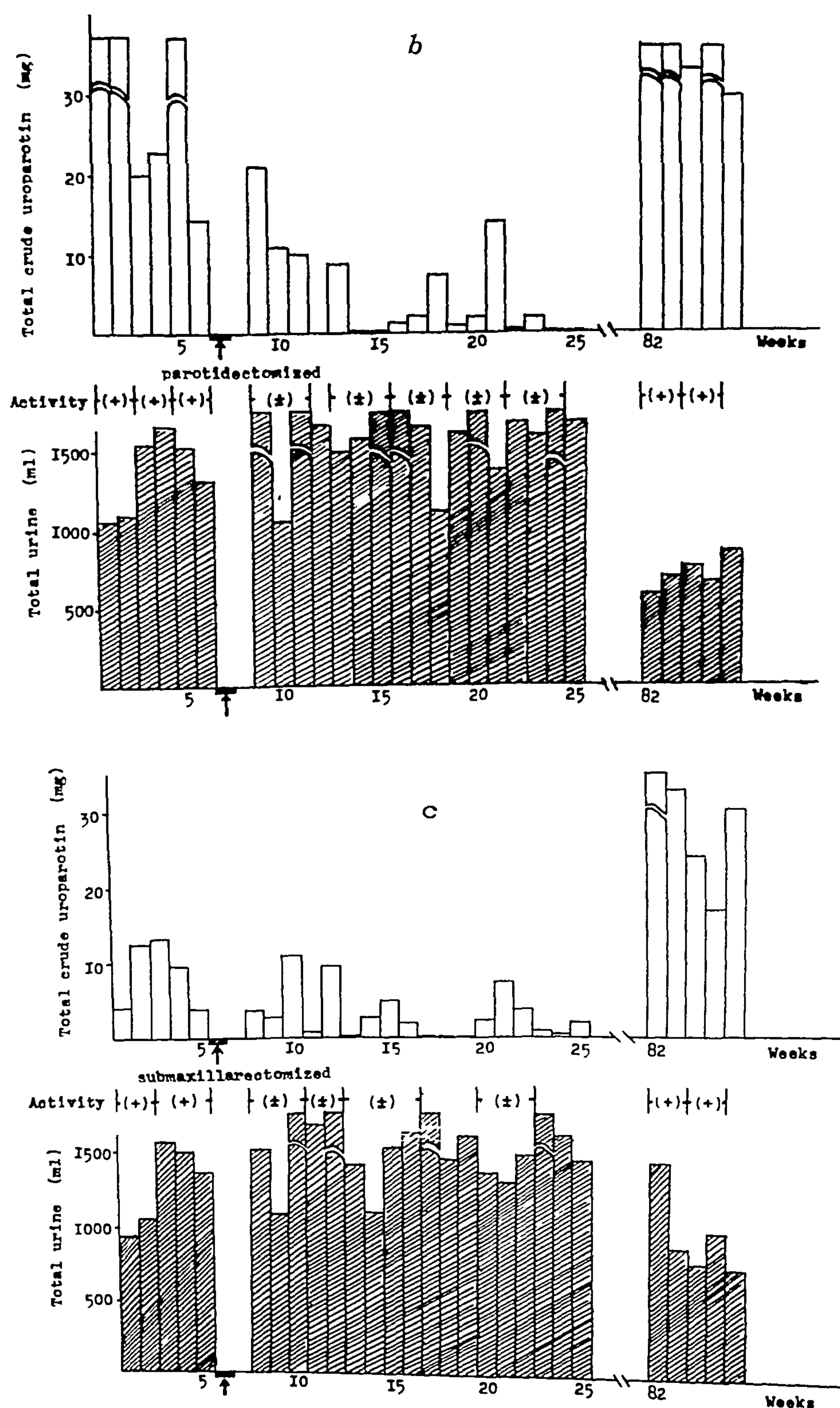


FIGURE 64 (*b*) Effect of parotidectomy on rabbit uroparotin Parotid gland was ectomized in the seventh week (*c*) Effect of submaxillarectomy on rabbit uroparotin Submaxillary gland was ectomized in the sixth week

Excretion of Uroparotin in Salivary Glandectomized Animals

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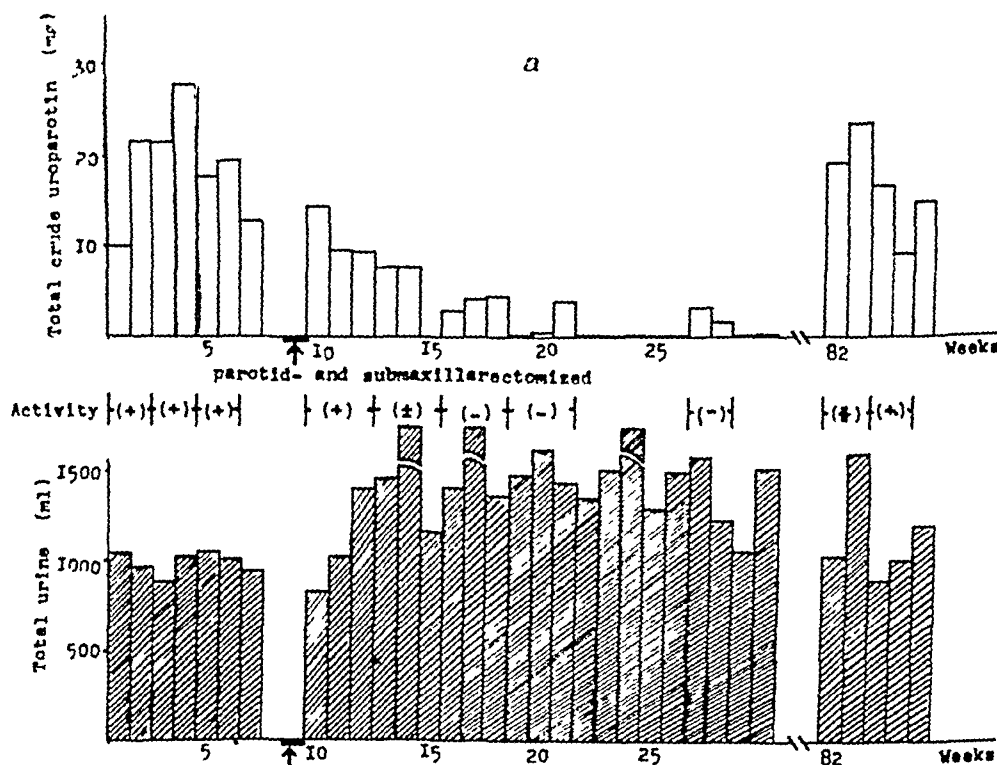


FIGURE 64 (*a*) Effect of parotidectomy and submaxillarectomy on rabbit uroparotin. Both parotid and submaxillary glands were ectomized in the ninth week. Minimum doses affecting activities: ++, <0 5 mg /kg ; +, 1 0 mg /kg , ±, 2 0 mg./kg.; –, >4 0 mg./kg

it was assumed that the function of the salivary glands and the excretion of uroparotin may be closely related.

Chemical Properties[83]

Uroparotin was found to be almost homogeneous, by ultracentrifugal as well as electrophoretic analysis in several types of buffer solutions (FIGURES 65 and 66). The isoelectric point of both α- and β-uroparotin was calculated to be *p*H 2.9, from results of paper electrophoresis in which bovine serum albumin was used as control and dextran for correction (FIGURE 67).

Two-dimensional and circular paper chromatography revealed the following seventeen amino acids in the hydrolyzates of α- and β-uroparotin: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine,

exist between such a serum glycoprotein and uroparotin having a rather low isoelectric point.

Carbohydrate Moieties in the Uroparotin Molecule[86]

Qualitative analysis was conducted with the sulfuric acid hydrolyzates of α- and β-uroparotin. The following color reactions were used for the pre-

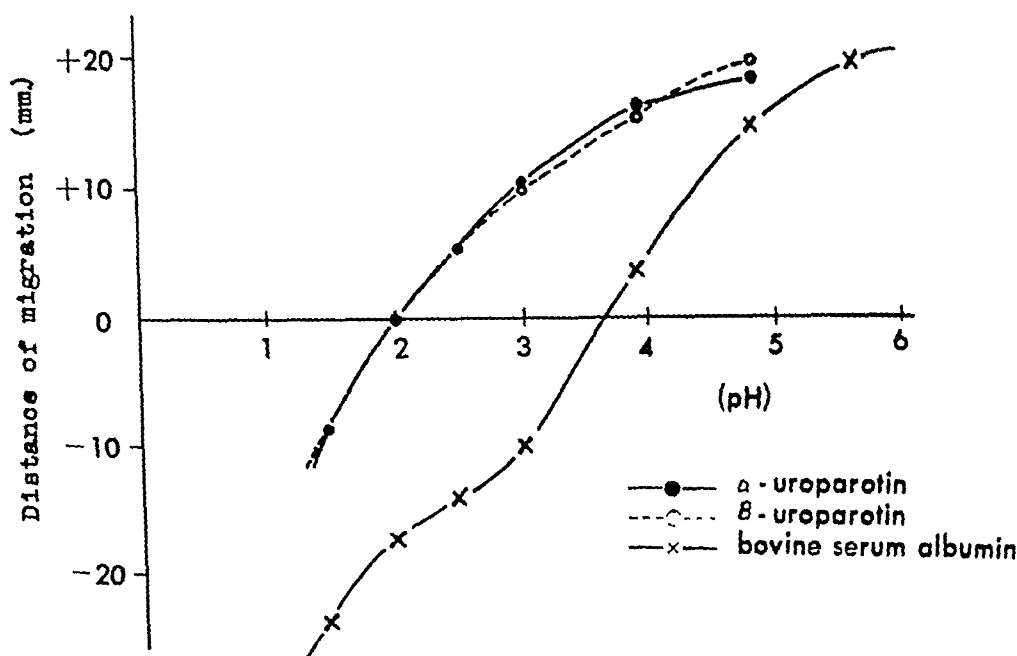


FIGURE 67. Migration of α- and β-uroparotin and bovine serum albumin as a function of pH.

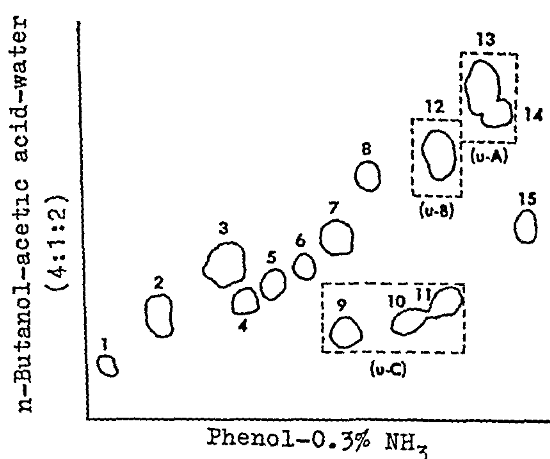


FIGURE 68 Two-dimensional paper partition chromatogram of uroparotin hydrolyzate with hydrochloric acid 1, Cys, 2, Asp; 3, Glu; 4, Ser, 5, Gly; 6, Thr, 7, Ala; 8, Tyr; 9, Lys; 10, His, 11, Arg, 12, Met (Val), 13, Leu (Isoleu), 14, Phe, 15, Pro.

liminary analysis of carbohydrate moieties: orcinol, anthrone, and cysteine reactions for hexose, Bial's and Tollen's methods for pentose; the Elson-Morgan reaction for hexosamine; the naphthoresorcinol reaction for uronic acid; and Bial's method[84] for sialic acid. From these experiments it was

leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and tyrosine (FIGURES 68 to 70).

Some other physicochemical and chemical properties are listed in TABLE 29. As shown in TABLE 30, uroparotin is considered to be a glycoprotein, on the

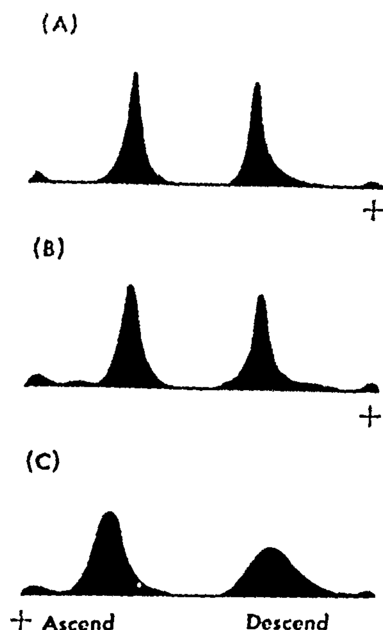


FIGURE 65. Electrophoretic patterns of uroparotin A: pH 8 6, μ = 0.1, Veronal buffer, 8 mAmp., 45 min.; B: pH 5.1, μ = 0 1, acetic acid buffer, 8 mAmp., 45 min.; C: pH 2 0 μ = 0 2, glycine buffer, 8 mAmp , 60 min.

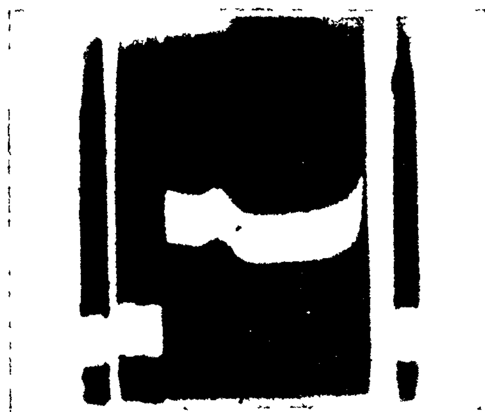


FIGURE 66 Ultracentrifugal pattern of uroparotin (pH 7 8 phosphate buffer, 59,780 rpm, 45 min)

basis of its color reactions. It is interesting that sialic acid is detected as a component of carbohydrate moieties. Odin and Werner[84] and Schmid[85] have reported the isolation of glycoprotein containing sialic acid from human serum, and it has been pointed out that the low pH of the isoelectric point of the substance is due to the sialic acid present. Therefore, a relationship may

Paper chromatography for sialic acid was carried out on the sulfuric acid hydrolyzate of uroparotin. The reference sialic acid was prepared from porcine submaxillary gland by the method of Blix *et al.*[87] Sialic acid was detected by spraying orcinol-trichloroacetic acid reagent. Porcine sialic acid revealed a

TABLE 29
PROPERTIES OF UROPAROTIN

Property	α-Uroparotin	β-Uroparotin
Elementary analysis	C, 44.24%, H, 7.05%; N, 12.64%, P, 0.22%, ash, 3.56%	N, 11.9%; P, 0.20%; ash, 2.52%
Isoelectric point	*p*H 2.9	*p*H 2.9
$E^{1\%}_{1\,cm}$ 277 mμ, *p*H 6.9 phosphate buffer	9.2	8.6
$E^{1\%}_{1\,cm.}$ 288 mμ, 0.1 *N* NaOH	10.6	10.3
Amino acid component	17 acids isoleucine and those in bovine parotin, less valine	17 acids: isoleucine and those in bovine parotin less valine
Tyrosine (%)	4.03	3.95
Tryptophan (%)	2.35	2.31
Color reactions		
For protein:		
Ninhydrin	+	+
biuret	+	+
Millon's	+	+
For carbohydrate.		
Molisch's	+	+
Orcinol	+	+
For sialic acid.		
Bial's	+	+
direct Ehrlich	+	+
diphenylamine	+	+
Lipidprotein test (Sudan black B reaction)	−	−
Phosphoprotein test	±	±
Solubility in 20% TCA	+	+
Solubility in 1.8 *M* perchloric acid	+	+

TABLE 30
PRELIMINARY COLOR REACTIONS OF CARBOHYDRATE COMPONENTS IN UROPAROTIN

Sugar	Reaction	Effect	
		α-Uroparotin	β-Uroparotin
Hexose	Orcinol	+	+
	Anthrone	+	+
	Cysteine	+	+
Pentose	Bial's	−	−
	Tollen's	−	−
Hexosamine	Elson-Morgan	+	+
Uronic acid	Naphthoresorcinol	−	−
Sialic acid	Bial's	+	+

found that hexose, hexosamine, and sialic acid were present in both α- and β-uroparotin; neither pentose nor uronic acid were detected (TABLE 30). Paper chromatography of the hydrolyzate of uroparotin with various solvents was used to detect individual carbohydrate components. From these experiments it was confirmed that mannose, galactose, and galactosamine were present in both α- and β-uroparotin. However, neither fucose nor uronic acid could be detected definitely.

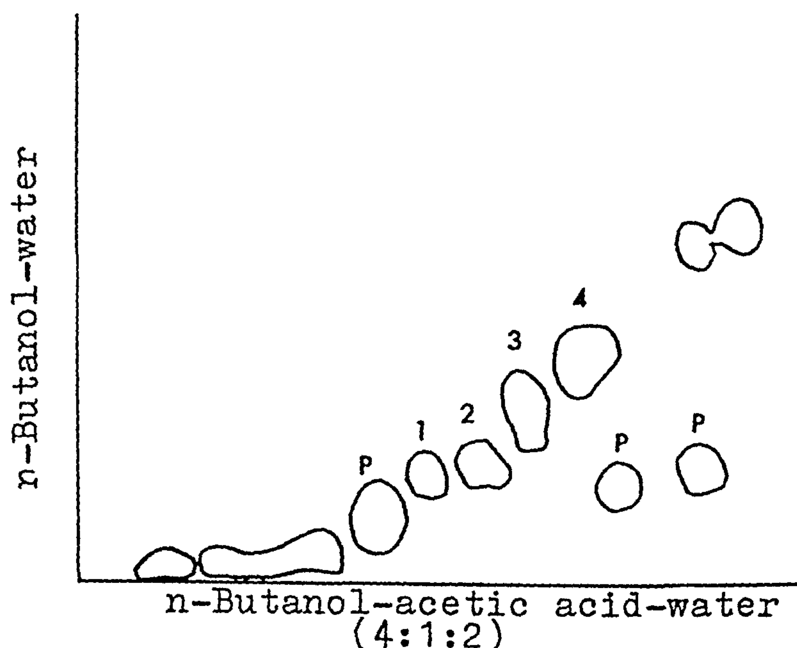


FIGURE 69 Two-dimensional paper partition chromatogram of uroparotin hydrolyzate with baryta. 1, Pro; 2, Tyr; 3, Try, 4, Met; 5, peptides

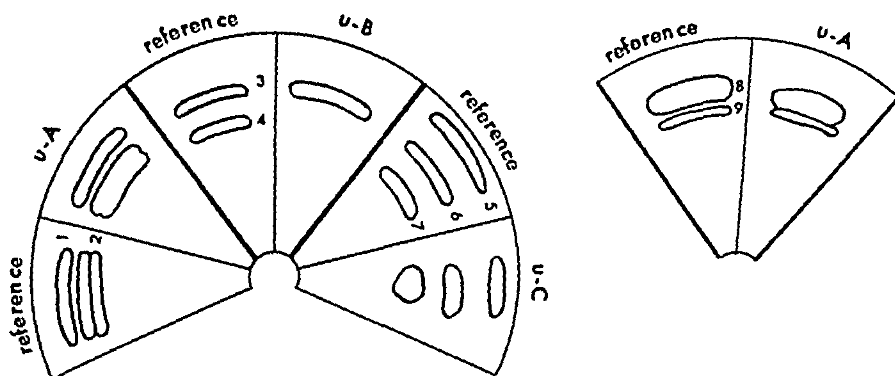


FIGURE 70 Circular paper partition chromatogram of uroparotin hydrolyzate. Sample solutions from U-A, U-B, and U-C were applied on Whatman No 1 filter paper Upper part was developed with *s*-collidine-2,6-lutidine-water (1:1:1) solvent, diethylamine was added, pyridine-acetic acid water (50:35:15) was added to the lower part. The spray used was Ninhydrin reagent, and the reference amino acids were as follows 1, phenylalanine (greenish blue), 2, leucine + isoleucine (purple); 3, methionine (brown), 4, valine (purple); 5, histidine (purple); 6, arginine (greenish blue), 7, lysine (purple), 8, phenylalanine + leucine (purple), 9, isoleucine (blue-purple).

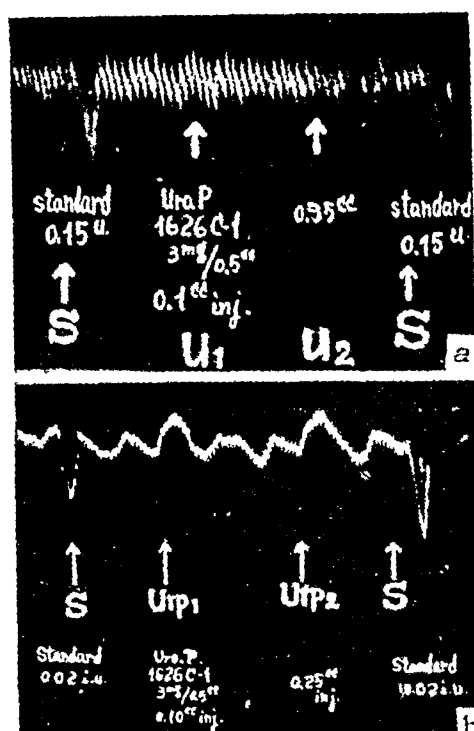


FIGURE 71. Effect of uroparotin on dog and chicken blood pressure (*a*) Dog. S· kallikrein standard (0 15 I U.); U_1, uroparotin (0 6 mg); U_2, uroparotin (2.1 mg). (*b*) Chicken S: Oxytocin standard (0 02 I U), Urp_1, uroparotin (0 6 mg); Urp_2, uroparotin (1 5 mg). After injection of uroparotin a slight rise in blood pressure was observed

TABLE 32
PROTEOLYTIC AND OTHER HORMONAL ACTIVITIES OF UROPAROTIN

Action	α-Uroparotin	β-Uroparotin
Pepsin*	less than $[Pu]^{cas\ 275}_{\gamma\text{-tyr}} = 2.2$/mg.	Identical
Trypsin†	less than $[Pu]^{cas\ 275}_{\gamma\text{-tyr}} = 3\ 5$/mg.	Identical
Kallikrein (2 1 mg.)	None	None
Oxytocin (1.5 mg.)	None	None
Corticotropin‡	less than 0 01 I U./mg.	Identical
Gonadotropin§ (1 mg. × 3 days)	None	None
Luteotropin‖ (1 mg × 3 days)	None	None

* Crystalline pepsin· $[Pu]\frac{cas\ 275}{\gamma\text{-tyr}} = 900$/mg

† Crystalline trypsin· $[Pu]\frac{cas\ 275}{\gamma\text{-tyr}} = 1500$/mg. The proteolytic activities were expressed in terms of γ-tyrosine released in a minute

‡ According to Sayer's method using Schering-ACTH as the standard preparation.

§ Tested by rat ovary weight method.

‖ Pigeon method was used for the estimation of lactogenic action.

faint spot, possibly due to the presence of a small amount of its methyl ester, in addition to the main spot due to its N-glycolyl form. The spot of sialic acid from uroparotin did not correspond to either spot of porcine sialic acid. From these results it may be hypothesized that sialic acid in uroparotin contains an N-acetyl group.

Quantitative analysis of the carbohydrate components of both α- and β-uroparotin was carried out by the following methods. The amount of hexose in unhydrolyzed uroparotin was determined by the orcinol,[88] anthrone,[89] and cysteine[90] methods. Hexosamine in the hydrolyzate of uroparotin was determined by means of the modified Elson-Morgan method.[91] For the determination of sialic acid, uroparotin was treated according to the procedure described by Svennerholm,[92] namely, hydrolysis with 0.1 N sulfuric acid in a sealed tube at 80° C. for 1 hour, and purification by column chromatography (Dowex-2) to remove the contamination by other carbohydrate components. The determination of sialic acid was carried out according to both Bial's and Ehrlich's

TABLE 31
RESULTS OF QUANTITATIVE DETERMINATION ON CARBOHYDRATES IN α- AND β-UROPAROTIN

Sugar	Method	Carbohydrate in α-uroparotin (%)	Carbohydrate in β-uroparotin (%)
Hexose	Orcinol	11.8	12 8
	Anthrone	11.2	10.8
	Cysteine	14.3	13 5
Hexosamine	modified Elson-Morgan	5.3	5.6
Sialic acid	Bial's	4.6 (6.0)*	4 2 (5.6)
	direct Ehrlich	4 3 (5.5)	4 8 (6.2)

* Corrected values in parentheses.

direct methods,[93] with porcine (N-glycolyl) sialic acid as the reference. The results are shown in TABLE 31, where the figures in parentheses are values corrected to pertain to N-acetyl sialic acid.[94]

Amounts of each carbohydrate component found in α- and β-uroparotin were similar. Therefore, the marked difference between biological activities of the two types of uroparotin could not be attributed to differences in carbohydrate components.

Biological Properties

As shown in FIGURE 71, uroparotin did not decrease the blood pressure in a phenobarbital-anesthetized dog. Therefore, uroparotin is not identical with kallikrein, even though the latter has also been reported to occur in urine[95] Similarly, oxytocic and other hormonal actions were not observed in uroparotin (FIGURE 71 and TABLE 32).

Pepsinogen (uropepsin) was reported to be similar to uroparotin in that it appeared in human urine with seasonal variation in activity.[96,97] However, uroparotin differs from uropepsin, since the former exhibited no proteolytic activity as measured by the method of Kunitz.[98]

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SUMMARY

T. Ogata presented the hypothesis that the salivary gland incretes a hormone activating the mesenchymal tissues. The parotid gland is said to play a principal role in this endocrine function, the submaxillary gland to cooperate with it, and the sublingual gland to be nonparticipating.

My associates and I succeeded in isolating an active principle from the bovine parotid gland in a pure crystalline form, and named it parotin; it was verified that parotin is able to compensate experimental animals and humans for deficiency of the parotid gland. Subsequently my associates and I isolated parotinlike substances from cattle submaxillary glands, from human saliva, and from human and rabbit urine, and named them S-parotin, saliva parotin A, and uroparotin, respectively. All these active principles are protein in nature. Parotin has been determined to have a molecular weight of about 132,000 by ultracentrifugal analysis and of 128,000 by light scattering.

Parotin and parotinlike substances are biologically active in lowering the serum calcium level in rabbits (calcium activity), in decreasing and subsequently increasing the number of circulating leukocytes in rabbits (leukocyte activity), and in promoting the calcification of incisor dentine in rabbits and rats (dentine-calcification activity). These activities were used for bioassay of parotin and parotinlike substances, calcium activity for quantitative estimation, and the other activities for qualitative identification.

Methods of isolating parotin and parotinlike substances, physicochemical properties, chemical and biological natures, and specific biological activities are described.

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crine role of the salivary glands are not altogether convincing, since the removal of salivary glands results in a loss of a turnover mechanism for electrolytes and minerals that may have a direct effect on the systemic mineral metabolism. It is also possible that sialoadenectomy has an indirect effect on the function of other endocrine glands affected by such shifts in mineral or electrolyte balance.

In relation to the biological effects involved, we have had the opportunity to test the effect of parotin on the serum calcium of intact and sialoadenectomized rats; the results of this study are summarized in TABLE 1. We could

TABLE 1
THE EFFECT OF PAROTIN ON THE SERUM CALCIUM OF INTACT AND SIALOADENECTOMIZED RATS
Male Rats (140 gm.)

Ten animals per group	Calcium mg % ± S.E
Group 1, controls	11.3 ± 0.4
Group 2, controls + parotin	11.5 ± 0.3
Group 3, operated	10.9 ± 0.4
Group 4, operated + parotin	11.3 ± 0.3

Parotin was given in 6 divided doses over 48 hours (total dose 10 mg./kg.). Animals exsanguinated 6 hours after last dose.

TABLE 2
THE EFFECT OF PARATHYROID EXTRACT ON INTACT AND SIALOADENECTOMIZED RATS
Male Rats (150 gm.)

Ten animals per group	Calcium mg. % ± S E.
Group 1, controls	10 8 ± 0.2
Group 2, controls + PTE	14 4 ± 0.3
Group 3, operated	11.0 ± 0.3
Group 4, operated + PTE	18.4 ± 0.3

Two hundred units of parathyroid extract was given in divided doses over a 24-hour period. Animals were exsanguinated 6 hours after the last injection.

not detect any significant decrease in the serum calcium of the treated animals compared to the control groups. It is possible that the serum calcium-lowering effect of parotin may not be common to all laboratory animals.

We have been interested in the possible relationship between the salivary glands and the parathyroids in relation to calcium homeostasis. Removal of the salivary glands of rats on a standard laboratory diet appears to alter the serum calcium response to parathyroid extract.[2] TABLE 2 summarizes the results obtained in these studies. There is a significant increase in the serum calcium response to parathyroid extract in the sialoadenectomized rat in the time period studied. The salivary glands may conceivably elaborate a substance antagonistic to parathyroid extract. It may be that the salivary glands, like the liver, inactivate parathyroid extract. On the other hand, the absence

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Discussion of the Paper

LEON KRAINTZ, *University of Texas, Dental Branch, Houston, Texas:* One of the duties of the investigator is to keep an open mind to new and even unorthodox concepts. The studies on parotin presented by Yosoji Ito represent over a decade of work in Japan and fill a gap in the needed exchange of information and ideas, since very few investigators in the United States are familiar with these studies. I had hoped that Ito would present more documentation proving that parotin is indeed a hormone. Had parotin been referred to as an extract or even as a factor, as in the case of a similar problem presented elsewhere in this monograph by Levi-Montalcini, the parotin studies would have been evaluated differently. The presentation of control studies using extracts of other tissue prepared in the same manner as parotin would have provided more convincing evidence than all the extensive studies done with parotin alone.

It is difficult in the allotted space to comment critically on all the material presented, since it represents a review of work done by many investigators other than Ito. I should like, however, to single out one of the studies cited, which presumably adds evidence to the hormonal nature of parotin. The studies on the localization of I^{131}-labeled parotin, indicating that it is selectively localized in the liver and spleen, in no way proves that these tissues are target organs of parotin, such localization indicates only that the reticuloendothelial system is active in removing this administered protein from the circulation. Other radioiodinated protein macromolecules would also localize in these tissues. The use of radioiodinated serum albumin for control distribution studies indicates only the amount of residual plasma retained in an organ and is not an adequate control for localization of radioactive-labeled protein hormones This same criticism would apply equally to other published studies on target-organ localization by radioiodinated protein hormone preparations.[1]

Admittedly, the salivary glands may play a role in calcium metabolism and homeostasis through their exocrine functions Details concerning the endo-

THE EFFECT OF PAROTIN IN MICE

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Introduction

Data to support concepts that the salivary glands are activated by endocrine systems or are in part endocrine organs have been reported for more than 70 years. First to present such a concept was Hardin[1] in 1886, who advanced the hypothesis that salivary glands participated as end organs to endocrine stimulation. This conclusion was reached because of the painless bilateral swelling of the parotids of a patient during each of six successive pregnancies. More recently Phillips[2] reported a similar activity of the parotids in a patient during two successive pregnancies and lactation periods.

In 1935 Ogata proposed a salivary gland endocrine theory.[3] He[4,5] and others,[6-8] experimenting with animals, observed that the active principle of extracts of the salivary glands of young beef cattle provided a hormonelike substance that they called parotin. This substance consistently lowered the serum calcium levels in rabbits by approximately 15 per cent. Enhancement of the calcification of teeth and bones was another important effect resulting from its administration. When parotin was administered to rats, there was a greater uptake of labeled inorganic phosphorous (P^{32}) in the incisors and femurs.[9]

In addition, it has been observed that parotin activates the adrenocortical function of the pituitary,[10] which is significant since it has been established likewise that a hormonal relationship exists between the pituitary and the salivary glands.[11,12] This connection controls the morphology of the salivary glands and their duct systems.

A substance has also been obtained from human saliva[13] and from the urine[14] of healthy young men that has an active principle like that of parotin.

While the foremost relationship of extracts of the salivary glands concerns skeletal growth and mineralization of the teeth, there are other *in vivo* activities that have yet to be investigated.

It is the purpose of this report to present some observations concerning parotin. This report will include some findings regarding the femurs and incisor teeth of mice, as well as observations concerning changes in the testes and ovaries when parotin is administered.

Materials and Methods

The mice used for this part of the experiments with parotin were predominately young adult DBA Rocklands (60 to 90 days old) of both sexes. Some young adult Swiss mice were also used. In addition, old male and female Swiss mice (past breeding) were injected intramuscularly with parotin. Injections were usually given on alternate days until, over a 30-day period, 10 injections had been administered. In certain other instances these injections were given daily until a total of 10 injections had been given. All the old Swiss mice and some of the young Swiss and Rocklands received injections in

of the continuous cycling of calcium from blood to saliva through the gut may be responsible for this effect of sialoadenectomy on the serum calcium response to parathyroid extract.

The studies made by the Japanese workers on the salivary glands and the possible hormonal effects of salivary gland and saliva protein preparations have emphasized the role of the salivary glands in calcium metabolism and should stimulate much needed research in this field.

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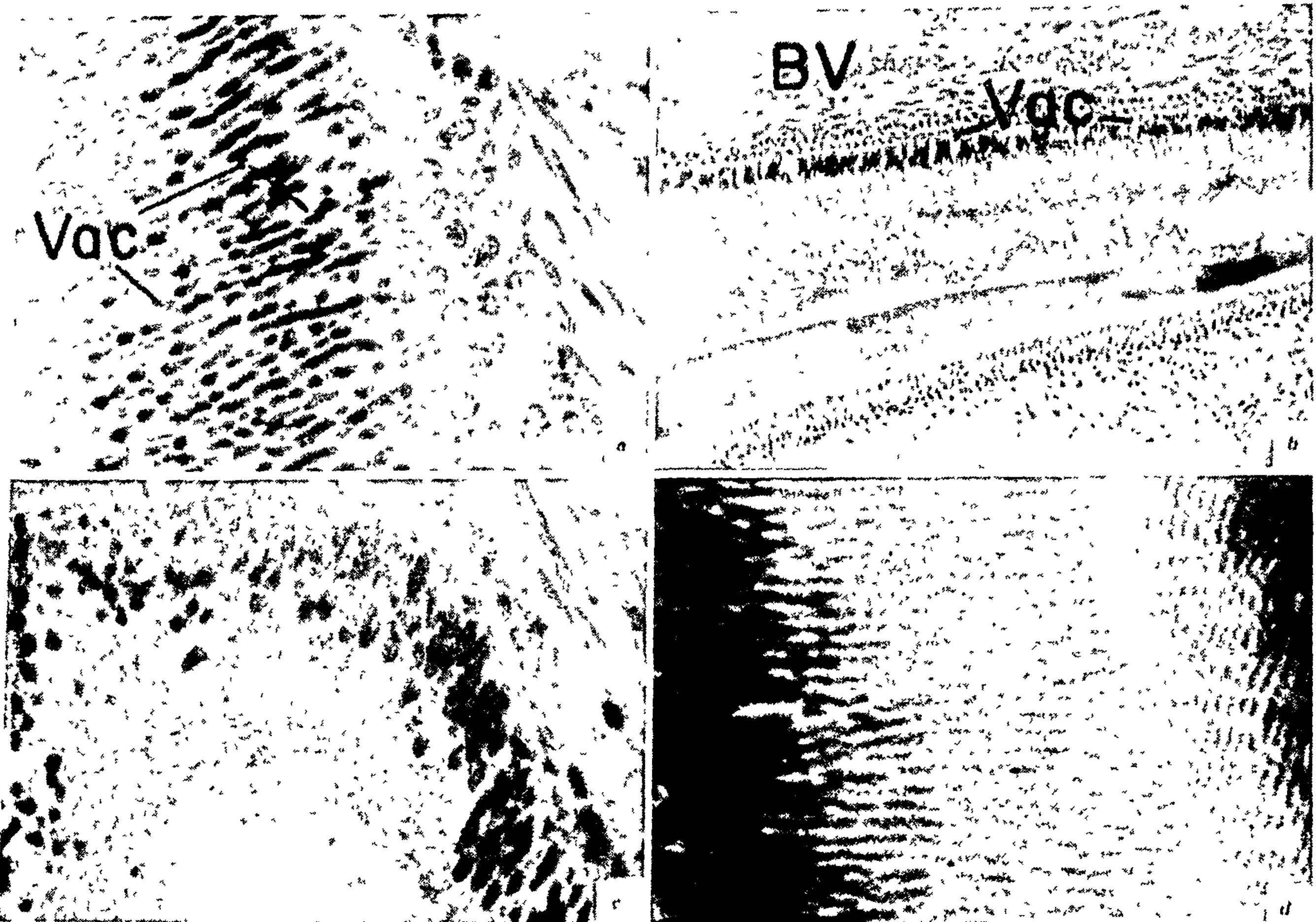


FIGURE 1 Enamel protein matrix in mandibular incisors of Rockland mice· (a) female given 2 injections of 0 30 mg parotin over 6 days (×1068); (b) female given 7 injections of 0 30 mg. parotin over 15 days (×295), (c) male given 6 injections of 0 15 mg parotin over 13 days (×1068); (d) male, normal control (×1068) BV, blood vessels, Vac, vacuoles

this manner. In all instances animals were sacrificed intermittently. Animals were weighed before treatment and again before sacrifice. Three milligrams of parotin was dissolved in 2 cc. of normal saline, and injections were given in the amounts of one fifth and one tenth cc. of this solution. A total of 350 male and 350 female Rocklands was treated, along with 60 male and 60 female young Swiss mice and also 40 male and 40 female old Swiss mice. In each series that was injected, adequate controls were taken, and some animals received sham injections. Diets were Purina Rat Chow and water ad libitum.

Sacrifice was made by chloroform inhalation, and particular tissues or organs were recovered and immediately fixed for histologic study. Jaws and femurs were immediately freed of adhering flesh and placed in Zenker's solution for 12 hours. They were then removed and washed in running tap water for 24 hours before they were placed in 80 per cent alcohol for another 24 hours. Then they were decalcified. The ovaries, testes, adrenals, pancreases, and salivary glands were fixed in formalin. All tissues were paraffin-blocked and cut at 7-μ thickness. Hematoxylin and eosin was routinely used for staining, but other stains were also used. Testes were weighed at sacrifice and weight comparisons were made with the controls.

In this report, findings on the epiphyseal plates and marrow spaces of femurs and the zones of enamel secretion in the mandibular incisors are considered, as well as findings on the testes and ovaries.

Results

Enamel-Secreting Zones of Mandibular Incisors

Marked alterations were noted in the secretory ameloblasts and the newly formed enamel matrix of mice given 0.30 mg. of parotin at each injection. These disturbances were evident in females as well as males. When 4 injections of 0.15 mg. of parotin were given on alternate days, changes were noted in the cytoplasm of the ameloblasts as well as in the compartmental matrix formations FIGURE 1*a* represents an area of a mandibular incisor from a female Rockland mouse given two 0.30-mg. injections of parotin in 6 days. Striking disturbances are not yet apparent, but vacuoles are found between the ameloblasts whose cytoplasm has become more granular. No changes were apparent in the enamel protein matrix, but adjacent blood vessels were engorged with red blood cells. This was likewise true of the pulpal vessels of this incisor tooth.

FIGURE 1*b* shows the secretory area of the enamel matrix in the mandible of a female Rockland mouse. Seven injections of 0.30 mg. of parotin were given over a period of 15 days. Severe distortions in the secretory portions of the ameloblasts are noted, and it is apparent that the Tomes's processes have been unable to deliver the protein matrix in any consistent pattern. Large vacuoles lie between the ameloblasts and seem to push them aside. These vacuoles were also plentiful in adjacent areas and in the odontoblast layer of the pulp. Another important finding was the marked vascular activity Blood vessels in the incisor region were increased both in size and in number and were closely packed with red blood cells

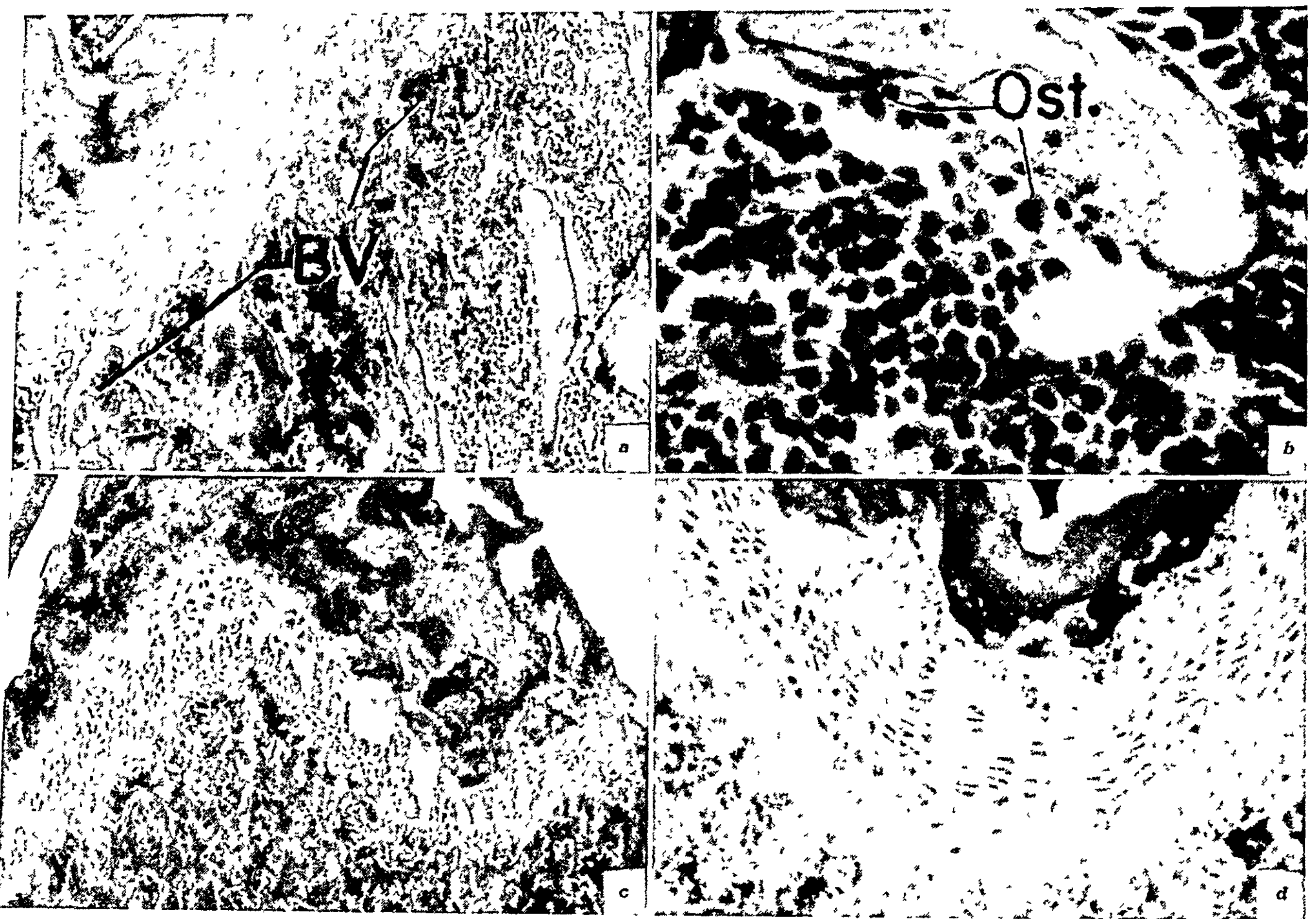


FIGURE 2. Epiphyseal plate, femur, in Rockland mice. (*a*) male given 5 injections of 0 30 mg. parotin over 15 days (×295); (*b*) female given 7 injections of 0 15 mg. parotin over 22 days (×1068); (*c*) male given 4 injections of 0.30 mg parotin over 9 days (×295); (*d*) male, normal control Ost, osteoclasts.

The above-mentioned changes were not as striking when the dosage was 0.15 mg. of parotin per injection. The enamel matrix formed in a more orderly fashion, and alterations in the ameloblasts were not pronounced. Increases in vascularity and the number of vacuolar areas between the ameloblasts were the most persistent phenomena. Considerable irregularity in the degree of calcification was often evident in the newly formed enamel layer. Shown in FIGURE 1*c* are disturbances at the growth center of the ameloblasts of a male Rockland mouse that received 6 injections of parotin at 0.15 mg. over 13 days. FIGURE 1*d* represents the normal control of this area from an untreated male Rockland mouse

Epiphyseal Plates of Femurs and Their Marrow Spaces

These areas showed remarkable changes following parotin administration. The increase in the size and number of vessels at the metaphyseal border, as well as within the marrow spaces, was most striking. Along the border of the metaphyses, numbers of free red blood cells were also found. Some of these changes in a male mouse that received five 0.30-mg. injections of parotin over a period of 15 days are demonstrated in FIGURE 2*a*. Osteoclastic activity is apparent in many places. Numbers of giant cells can be distinguished from megakaryocytes within the marrow spaces of the femurs. This is depicted in FIGURE 2*b* showing the epiphyseal plate of a female Rockland mouse that received seven 0.15-mg. injections of parotin over 22 days. The area is a marrow space above the terminal plate. In addition, there is a dramatic upset in the sequence of events taking place in the epiphyseal plate depicted in FIGURE 2*c*. This animal, a male Rockland, received 4 injections of parotin at 0.30 mg per dose over 9 days. The chondrocytes toward the metaphyseal border have failed to degenerate. A decrease in their proliferation and in some areas ballooning, instead of degeneration, are seen. In many places there appears to be a reversion of these cells, perhaps to precursors of connective tissue cells. Where parotin was given, the epiphyseal plates appeared reduced in thickness, and osteoid proliferations were often found deep within these areas. FIGURE 2*d* shows the area of the epiphyseal plate from a normal control male Rockland mouse.

The Testes and Parotin

During the course of these experiments it was noted that adult male mice (Rocklands) receiving parotin injections became increasingly vicious and hard to handle as the injections progressed Upon further examination it was noted that the position of the testes was altered as compared to the control animals. Grossly, the scrotal area was pinkish and the testes seemed more prominent than those noted in the untreated mice Upon histologic examination it was evident at once that cellular activity of the seminiferous epithelium was more pronounced in parotin-injected animals. Because of this activity, the lumens were smaller in the treated than in the untreated males The growing layers of cells of the early pachytene or transitional stage were increased and the cells were larger, in animals given parotin. FIGURE 3*b* represents an area of the seminiferous tubules from a Rockland mouse that received nine 0.30-mg. in-

jections of parotin over a period of 10 days. A similar area from a control mouse (Rockland) is shown in FIGURE 3*a*. Both mice were 90 days old at the start of these experiments and had almost identical body weights. Except for occasional heavier testes among the control animals, the testes of parotin-injected animals were consistently heavier by 20 to 25 per cent. Spermatids were found in larger groups in the parotin-treated animals. Numbers of these spermatozoa nursing at the Sertoli cells were greater in parotin-treated than in untreated mice. The effect of parotin in old Swiss mice (past breeding) is shown by comparison of FIGURES 3*c* and 3*d*. FIGURE 3*c* represents a testis from an untreated mouse. The region depicted by FIGURE 3*d* represents the results after nine 0.30-mg. injections of parotin were administered over a 15-day period. Note the large blood vessel, a consistent feature. Following parotin injections there was considerably more cellular activity throughout the seminiferous tubules in these old animals. The lumens of the tubules were smaller and cells at the early and late pachytene stages were larger and more numerous in the treated than in the untreated mice of this series, although these differences were seen occasionally also in the nontreated old males. Immature spermatozoa were seen in greater numbers of parotin-injected than in non-treated males. The blood supply was likewise greater in the treated animals. This observation coupled with the intertubular activity, the weight gain, and the increased numbers of immature spermatozoa were the most striking observations.

FIGURE 4*a* represents an ovary from an old Swiss mouse. FIGURE 4*b* is also an ovary from an old Swiss mouse, but in the latter instance 9 injections of parotin were given at 0.30 mg. per dose over 12 days. The increase in the vascular supply was the most striking feature in these treated animals. In young Rockland and young Swiss mice no definitive changes could be determined from counts of cell nests, follicles, corpora lutea, and corpora albicantia. Ovaries given varying injections were studied, and no progressive changes were noted. Counts were made in the ovaries of more than 200 treated and control mice, and no significant differences could be observed. Furthermore, there were no increases in the activity of the germinal epithelium. It would be interesting, however, to study breast tissue in female mice injected with parotin to determine whether any changes take place. Presumably, if increases in vascularity to these areas take place following parotin injections, there might likewise be alterations in the structure of these areas.

Discussion

Despite numerous investigations of interdependent occurrences taking place *in vivo* prior to and during the mineralization of teeth and bones, questions regarding the nature and functions of these occurrences have not yet been answered satisfactorily.[15,16]

These experiments indicate, however, that the enamel matrix formation can be altered by a series of parotin injections. This is true likewise for the series of events taking place in mice femurs, for parotin interfered with division of cartilage cells in the epiphyseal plates, as well as with their degeneration.

Some aspects of these experiments coincide with previous studies regarding

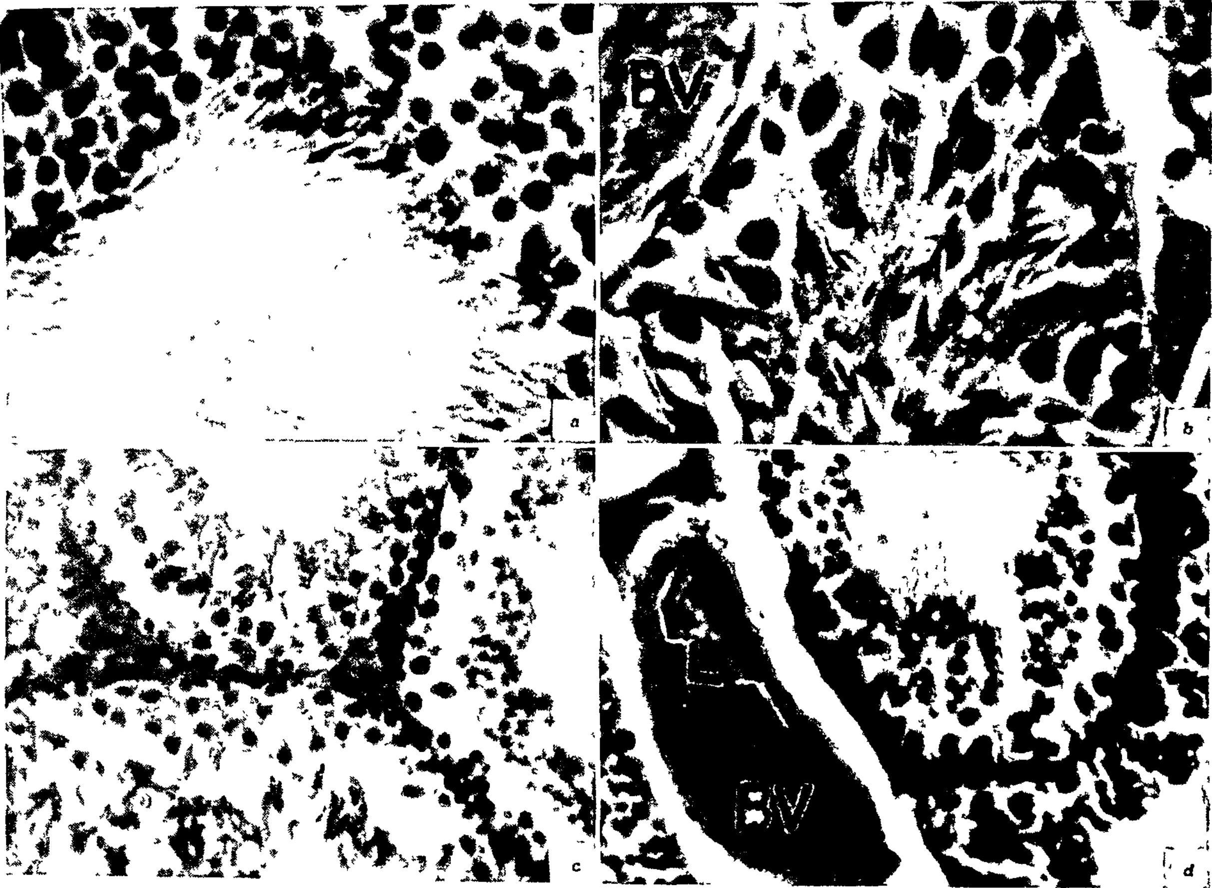


FIGURE 3. Testes in (*a*) Rockland mouse, control (×1068), (*b*) Rockland mouse given 9 injections of 0 30 mg parotin over 10 days (×1068), (*c*) old untreated Swiss mouse (×900), (*d*) old Swiss mouse given 9 injections of 0 30 mg parotin over 15 days (×900). L, leukocytes.

They obtained an enhancement of growth of mineralized tissues with rabbits that was not obtained with mice in the present experiments. The doses used could have equaled, in some instances, but never exceeded those used by the above-mentioned investigators. In previous experiments[18] none of the concentrations equaled that used by the experimenters referred to above.

Results in the present experiments cannot be compared with those obtained by Honjo *et al.*,[19] who found no changes in the epiphyseal plates of the tibias of young female rats injected with parotin. In one instance the dosage of 0.05 mg. per injection for 7 days was presumably not sufficiently high to cause changes in the epiphyseal plates of the control animals. This does not compare with dosages given in the present instance, nor with those given in certain previous experiments.[13,14,18] In a second instance, results on sialoadenectomized, hypophysectomized, and hypophysectosialoadenoctomized animals injected with higher amounts (0.50 mg. per injection) were not comparable. Perhaps a better comparison could be made if the original controls had received 0.50 mg. per injection instead of 0.05 mg.

Increases in vascularity were the most striking phenomena found in these studies. It is interesting to note that such vascular increases were not sex-specific. Particularly important is the fact that the striking increases in vascularity and blood volume in the femurs of animals could have raised the temperature of the marrow spaces, as suggested by Ito and Okabe.[13] It is possible that the increased osteoclastic activity in the femurs of animals receiving parotin was in some way a result of the increased circulation and accompanying marrow temperature rise, for this activity increase was significant.

This increase in vascularity is interesting likewise with regard to the testes in treated males, for it is well known that where there are cryptorchid testes, spermatic activity is decreased, presumably owing to an increase in ambient temperatures.[20,21] In the parotin-treated males, the young as well as the old, the activity of the seminiferous epithelium was much greater than that seen in untreated and/or comparative control animals. It was obvious that the vascular supply was increased both within the testes and in the surrounding connective tissue. Such a reaction would indicate that changes more subtle than a mere temperature rise due to an increase in the blood supply are involved in the stimulation of seminiferous tubules. It is significant likewise that in the present instances the reaction of parotin did not appear to affect the cellular activity of the interstitial cells similarly, for it is known that these cells can stimulate spermatogenesis.[22,23] Activity in these areas appeared confined to the blood vessels, and the interstitial cells did not appear to undergo any morphologic activities.

The activity of the ovaries was confined to the blood vessels and either was not as obvious or did not have the effect observed in other instances in these experiments. This was true for young as well as for older females.

Summary and Conclusions

Mandibular Incisor Teeth

These findings pertain to both male and female mice:

The secretory activity of the ameloblasts was interfered with when parotin was given to the mice at 0.30 mg. per injection.

the effect of CaF_2 on the incisors in mice.[17] In those studies, as in the present and other experiments[18] where 0.15 mg. of parotin was injected, it was found that CaF_2 widened the enamel matrix formation in the secretory zone A and



FIGURE 4. Ovaries in (*a*) old untreated Swiss mouse (×105), (*b*) old Swiss mouse given 9 injections of 0 30 mg parotin over 12 days (×105).

in the enlarged compression zone B, bringing it nearer to the base of the incisor. Numerous vacuoles appearing near and about the ameloblasts in the matrix secretory zones were present with both parotin dosages The effect was more marked where the higher dosages were used, however, and as a result the secretory activity of the ameloblasts was completely upset.

Ito and Okabe[13] and Ito *et al.*[14] used dosages stronger than were used in these experiments, on the basis of the dose per 100 grams of animal body weight.

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The formation of the enamel protein matrix was considerably altered when 0.30-mg. injections of parotin were given. Changes were less defined with 0.15-mg. injections; at times, however, considerable disturbances were seen.

The zone of compression, Zone B, in these incisors was similar to that obtained when mice were treated with CaF_2, and was nearer the base of the incisior. The secretory portion of the matrix, Zone A, was wider than usual.

The vascular bed at the growth center of these incisors was considerably increased in volume and number of vessels when parotin was administered.

Vacuoles appeared between the ameloblasts in the secretory Zone A, as well as in Zone B, when parotin was administered These vacuoles also lay in adjacent structures and were likewise found between the odontoblasts in the pulp.

Epiphyseal Plates of Mice Femurs

In both male and female there was marked increase of the vascularity along the terminal and metaphyseal plates. Marrow and trabeculae were crowded out by increases in blood vessels and blood sinusoids. Numbers of free red blood cells were found. Blood vessels encroached directly into the epiphyseal plates.

Cartilage cells did not degenerate, but in many instances appeared to undergo reversion. There was considerable ballooning and, in some places, the cartilage took on a fibrillar appearance. Cartilage plates were thinner than in the controls.

Osteoclastic activity was marked on both the proximal and distal borders of the epiphyseal plates. This was also true along the shafts of the femurs. Many multinucleated giant cells were found and were distinguishable from the megakaryocytes.

Testes and Ovaries

There was a marked increase in vascularity in both the testes and ovaries of animals that had received parotin.

Testes were usually 20 to 25 per cent heavier in the treated than in the untreated animals

There was considerably more activity in the early and late pachytene stages in the testes of animals given parotin Lumens of the seminiferous tubules were more crowded, and immature spermatocytes appeared in greater numbers around Sertoli cells.

Changes in the ovaries were not as obvious. No significant differences were observed between the treated and untreated animals with regard to egg nests, follicles at different stages, corpora lutea, and corpora albicantia

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Nerve-Growth Factors

In 1948 Bueker reported that fragments of mouse sarcoma 180, when transplanted to the body wall of three-day chick embryos, grow vigorously and are invaded by sensory nerve fibers from the adjacent spinal ganglia. The ganglia providing the innervation of the tumor are larger than the contralateral spinal ganglia that innervate the leg of the embryo.[10] Bueker compared these results with results obtained in previous experiments of transplantation of additional limbs in the thoracic segment of three-day chick embryos. In both groups of experiments the periphery was enlarged as a consequence of the transplant and, in both instances, the effect seemed to be restricted to the ganglia providing its innervation. Bueker concluded that nerve fibers branching in the transplant mediate the effect. an enlargement of the peripheral field of innervation therefore results in an enlargement of the sensory ganglia contributing nerve fibers to that field These experiments and our interpretation of the tumor effect have been reported by one of us in a monograph of The New York Academy of Sciences[11] and will therefore be only summarized here.

The tumor transplant in the body wall of 3-day chick embryos is invaded not only by sensory nerve fibers but also, to a major extent, by sympathetic nerve fibers. Sensory and sympathetic fibers adjacent to the tumor are highly hyperplastic and hypertrophic. Both effects far exceed the same effects called forth by transplantation of homologous chick tissue[12] The sympathetic ganglia that do not contribute to the innervation of the tumor are also hyperplastic and hypertrophic; they hyperneurotize the adjacent viscera and they even perforate the walls of blood vessels and branch freely into the lumen of large veins.[11,13] Similar effects were elicited by mouse sarcomas 180 or 37 transplanted onto the chorioallantoic membrane of 4- to 5-day chick embryos. In this position, the tumor and the embryo shared the circulation, but no direct contact was established between the neoplastic tissue and the nervous system of the host Such results could hardly fit into the scheme proposed to explain the results of previous experiments where the peripheral field of innervation and the associated nerve structures seemed to be linked to each other in a simple and linear proportion. We rejected this explanation of the tumor effect and suggested instead that the tumor produces and releases an agent that enhances the growth potentialities of sensory and sympathetic nerve cells[11,13] The years that followed were devoted to the chemical analysis of the tumor factor and to the investigation of its biological effects The results will be summarized in the following section.

The Tumor Factor

The chick embryo had provided a most favorable material for an investigation of the effects of the tumor on the nervous system of the host. The use of the silver techniques, in fact, had permitted one to trace the distribution of nerve fibers in the developing embryo and to follow the effects elicited by the tumor step by step from early developmental stages to hatching.[13] The embryo did not, however, provide an equally favorable condition for investigating the chemical nature of the active agent. Experiments with the tumor extract failed to replicate the effects elicited by the growing sarcomas and discouraged

EFFECTS OF THE EXTRACT OF THE MOUSE SUBMAXILLARY SALIVARY GLANDS ON THE SYMPATHETIC SYSTEM OF MAMMALS*

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When, in 1940, Lacassagne called attention to a sexual dimorphism of the mouse submaxillary glands, no reader of his two short communications[1,2] would have predicted the upsurge of interest in these glands that was to take place several years later. Lacassagne stated simply that there is a sexual dimorphism in the tubular portion of these glands and that this portion is more highly developed in male than in female mice. He then found that the situation is reversed when testosterone is injected into female and estrogen into male mice; these results indicated that the dimorphism is under the control of sex hormones.[1] In a following investigation Lacassagne reported that the tubular portion of this gland undergoes an even more severe atrophy in hypophysectomized mice, the normal condition is restored by administration of male hormones.[2]

These pioneer investigations by Lacassagne were extended by Raynaud and Rebeyrotte in France,[3] Grad and Leblond in Canada,[4] Junqueira and his coworkers in Brazil,[5-7] Suzuki in Japan,[8,9] and a number of investigators in the United States. Reports on more recent advances in the study of the correlations between the salivary glands and endocrine systems are included in this monograph.

Our interest in this problem was raised in a most unexpected way by a chance discovery that was to change the entire course of our research. For years we have been interested in the problem of differentiation and growth in the embryonic nervous sytem and in the search for agents that may influence these processes. This search was not extended to the salivary glands, since there was no indication in all the work mentioned above of any effect of the salivary glands on the differentiation of any sector of the nervous system. Neither was there any reference in the extensive literature on the nervous system to any influence exerted by oral tissues or, in fact, by any tissue on the growth potentialities of any type of nerve cell. The nervous system has only an insignificant representation among the tissues lodging in the oral cavity and no one would have suspected that the extract of rodent salivary glands could have a most powerful growth effect on some components of the nervous system. It would have been even more difficult to anticipate the opposite effect: the destruction of this same component by an antiserum to the salivary factor. Before describing our experiments and attempting an interpretation of the results, we shall outline briefly the experimental work that preceded and provided the background for this investigation.

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The photographs and photomicrographs were made by Cramer Lewis, Department of Illustration, Washington University Medical School, St Louis, Mo

sympathetic ganglia explanted *in vitro* as a fragment of mouse sarcoma or 15,000 μg. of the crude tumor extract. The two effects, in fact, are indistinguishable. The only difference, as one may infer from the above figures, is a quantitative one. The parallelism between the two nerve-growth agents extends also to their chemical properties. The activity of both agents is associated with a protein component that is heat-labile and nondialyzable. Both agents are destroyed by acid treatment and are stable to alkali treatment (0.1 *N* NaOH/hour at 26° C.) and to 6 *N* urea/hour at 0° C.[16,17] They have the same effects on the sensory ganglia of the chick embryo *in vitro*. Injections of microgram quantities of snake venom into the yolk of the developing embryo replicate the effects of mouse sarcomas: the sympathetic ganglia are many times larger than those of controls, and sympathetic nerve fibers flood the viscera and enter the blood vessels.[18]

The finding of two nerve-growth factors in two biologically unrelated sources, such as mouse sarcoma and snake venom, raised the question of the distribution of such agents in the animal kingdom, and prompted an extensive search for them. Since the snake venom is produced in modified salivary glands, it occurred to one of us (S.C.) to test the mouse salivary glands as a possible source of these agents. Although the mouse effect reported above had suggested other sources for the nerve-growth factor, we were certainly not prepared for the overwhelming confirmation given by the experiments that followed.

These experiments consisted of testing the effect of the extract of mouse salivary glands *in vitro* on the sensory and sympathetic ganglia of chick embryos. The results of these experiments and of those that followed are the principal object of the present report and are presented in some detail in the following pages.

The Salivary Nerve-Growth Factor

In vitro and in vivo effects on the chick embryo. Tissue culture provided again a most favorable method for testing the effects of the extract of mouse submaxillary salivary glands on the sensory and sympathetic ganglia of chick embryo. The addition of 1.5 μg./ml. of the crude gland homogenate evoked the same dense halo of nerve fibers from the explanted ganglia as did 6 μg. of the crude snake venom or 15,000 μg. of the sarcoma extract. Subsequent purifications of the salivary extract increased the potency of the active agent: 0.015 μg. of this fraction added to the medium of culture evoked a similar nerve-growth effect.[19]

The effect of the salivary gland extract was then tested *in vivo* by daily injections of microgram quantities in the yolk of 7- to 10-day chick embryos.[20] The embryos were sacrificed 3 to 4 days after the first injection and inspected for effects on the nervous and other systems. As we had anticipated on the basis of the *in vitro* results, we found that sensory and sympathetic ganglia were hypertrophic and hyperplastic, and that the viscera were flooded with nerve fibers. The size increase in the sensory ganglia far exceeded the results obtained with previous nerve-growth agents (FIGURE 1); sensory nerve bundles many times larger than those in controls branched beneath the skin and even perforated it and appeared at the free surface of the body (FIGURE 2). A more detailed analysis of this effect is planned.

further attempts along that line. We then decided to make use of the tissue culture technique. Sensory and sympathetic ganglia explanted from 7- to 9-day chick embryos were cultured *in vitro* in a medium consisting of 1 part of chicken plasma and 1 part of embryonic extract. In subsequent experiments the embryonic extract was replaced by a synthetic medium to obtain a more uniform nutrient. Three series of experiments were performed: in the first series the ganglia were combined with fragments of mouse sarcomas 180 or 37, in the second series the ganglia were combined with fragments of embryonic chick heart and, in the third series, the ganglia were combined with fragments of embryonic or fetal mouse heart. The cultures were incubated at 37° C. and inspected after 6, 12, and 24 hours. The ganglia of the first series produced a very dense halo of nerve fibers in the first 24 hours, whereas those of the second series produced in the same period very few fibers. Ganglia of the third series gave origin to a rather vigorous outgrowth of nerve fibers within the first day of culture. We concluded that mouse sarcomas elicit a potent nerve growth *in vitro* as well as *in vivo*.[14] The results of the third series suggested that "the sarcoma agent may be present in low concentration in normal mouse tissue."[14] Five years elapsed before the significance of this "mouse effect" was fully understood. In still another group of experiments, we tested the effects of other tumors *in vitro*. All tumors of mesenchymal origin proved to be more or less effective, while epithelial tumors such as mammary carcinomas and neuroblastomas were ineffective The two major results of these *in vitro* experiments were (1) evidence that mouse sarcomas enhance the growth potentialities of sensory and sympathetic ganglia even when isolated from the organism, and (2) that they provided us with a simple and rapid bioassay for the chemical analysis of the agent. This analysis was performed in the two following years. At first we tested the cell-free homogenate of the tumor. When it was found that it possesses the same activity as the growing tumor we investigated the effect of the different tumor fractions. A one-hundredfold purified "protein fraction" isolated from the tumor proved to possess the nerve-growth factor.[15] In an attempt to degrade the small amounts of nucleic acid present in the active fraction the material was incubated in the presence of a combination of nucleases and crude snake venom phosphodiesterase. In performing the experiment we anticipated either of two possible results a total loss of the activity due to the destructive action of the enzymes added, or no loss of activity if the protein but not the nucleic acids were the nerve-growth factor. Neither one of these two predictions was confirmed. The addition of the snake venom to the medium of culture had in fact increased the production of nerve fibers by the ganglia to an extent never before observed. The same results were obtained when the snake venom alone and not the tumor extract was added to the culture medium. Hence a third unpredictable alternative explanation presented itself. the snake venom harbored a very potent nerve-growth factor. Our efforts were then directed to the analysis of the biological and chemical properties of the snake venom factor.

The Snake Venom Factor

The addition of 6 μg./ml. of crude snake venom to the medium of culture, or of 0.15 μg. of the purified venom, has the same effects on sensory and

old and one was three and one-half months old. The results indicated that human ganglia respond to the salivary factor by producing the same dense halo of nerve fibers as do ganglia of birds and rodents. These results opened new perspectives and raised the question of whether the same effects would be produced in postnatal stages in mammals. To answer this question, we injected the active agent in newborn and adult mice.

In vivo effects on newborn and adult mice. In a preliminary group of experiments we injected a total of 25 mice with a fraction of the salivary extract that will be identified in this paper as fraction CM-1. This extract was obtained by precipitation of the active agent with ammonium sulfate and subsequent adsorption and elution through a cellulose ion exchange column (details of the technical procedure will be presented elsewhere[21]). This preparation was tested at first for its activity on sensory and sympathetic ganglia of chick embryo *in vitro*. One unit of biological activity is defined as the protein content per milliliter of tissue culture medium necessary to obtain a dense halo of nerve fibers within the first 24 hours of culture. The solution injected into newborn mice contained 6000 biological units/ml. It was injected subcutaneously in the amount of 0.05 ml./gm. of body weight. The injections were repeated daily from the first to the thirtieth day. Injected and untreated controls of the same litters were sacrificed every day or every other day and used for histological examination. Two main groups of effects were observed in all injected mice: (1) general somatic effects, and (2) specific effects on the sympathetic ganglia and their nerve fibers. Further experiments with a more highly purified extract, which will be identified in this paper as fraction CM-3 (details to be presented elsewhere[21]), resulted in the same effects on the sympathetic ganglia as those resulting from fraction CM-1, but in no somatic effects. We then suggested that the general somatic effects and the effects on the sympathetic ganglia are due to two different agents present in the salivary extract. The hypothesis was confirmed in subsequent studies. The two agents have different chemical as well as biological properties and they can be separated from each other (S.C.). It seems to be of considerable interest, in connection with the main topic of this conference, to consider both effects. At first we shall consider the somatic changes produced by the CM-1 fraction.

General Somatic Effects on Newborn Mice

Preliminary experiments indicate that the factor responsible for the general somatic effects is heat-stable but nondialyzable and precipitable with ammonium sulfate (S.C.). The daily injections of this extract resulted in the following changes, listed in the order of their first appearance. (1) loss of body weight and stunted growth; (2) failure of hair growth; (3) precocious opening of the lids; and (4) precocious eruption of the upper and lower incisors and calcification of these teeth in advance of those of controls

The loss of body weight was already apparent on the third day after birth and became progressively more severe in the following days. At the end of the fourth week the mice were barely larger than at the end of the first week (FIGURE 3*a*). The dwarf mice had lax and wrinkled skin in consequence of the almost total lack of subcutaneous fat. The failure of hair to grow became apparent at the end of the first week. At the end of the first month the mice were



FIGURE 1 Transverse sections through thoracic level of ten-day chick embryos *Arrows* point to ganglia (*a*) Control (*b*) Injected for 3 days with purified salivary extract; note increase of spinal ganglia in the injected embryo Silver-impregnated



FIGURE 2 Transverse sections through sacral level of embryos of FIGURE 1 Silver-impregnated (*a*) Control (*b*) Injected, *arrow*, sensory nerve increased in length, nerve perforates skin and appears at free surface of the body

In vitro effects on sensory and sympathetic ganglia of mammalian embryos. The purified salivary extract was then tested on sensory and sympathetic ganglia of mice and rat embryos In all instances it elicited the same nerve growth as in experiments with chick embryos. We then tested its activity on ganglia of human fetuses that had been delivered by hysterotomy for therapeutic abortion and immediately made available * Three were about two and one-half months

* We express our appreciation to Willard Allen, Washington University Medical School, who made this material available to us

still almost naked. The opening of the lids, which normally takes place between the twelfth and fourteenth days, occurred between the fifth and seventh days. The eruption of the lower and upper incisors and the first signs of their calcification preceded by four or five days these processes in controls (FIGURE 3*c* and *d*). All the above effects are surprisingly similar or, one may say, identical to the effects described by Field[22] in newborn rats injected with large doses of cortisone, (FIGURE 3*b*, *e*, and *f*). Experiments in progress by one of us (S.C) with cortisone-injected newborn mice resulted in similar effects, although the precocious opening of the lids was much less marked than with the salivary extract.

The striking parallelism between these two effects is expected to shed light on some still unknown function of the salivary glands. It will therefore be considered again in the *Discussion.*

Upon discontinuance of the treatment with the CM-1 fraction, the dwarf mice resumed growth, and their hair grew vigorously in the following months. At three months the injected and control mice could no longer be distinguished by gross inspection. The injection of the CM-1 fraction in a large group of adult mice for periods varying from one week to one month did not evoke any of the somatic effects described above. The effects on the sympathetic system of newborn and adult mice will now be considered.

Effects on the Sympathetic Ganglia in Newborn and Adult Mice

The CM-1 fraction was injected into 25 newborn mice; the CM-3 fraction was injected in the same amount in 12 newborn mice. An equal number of untreated controls of the same litters was made available. The effects of the CM-1 and of the CM-3 fractions on the sympathetic ganglia were of the same kind, although somewhat more pronounced when the CM-3 fraction was used. The difference may well be explained by the lack of toxicity of the CM-3 fraction, which did not exert any adverse effect on the injected mice: they were, in fact, as healthy and vigorous as the controls. Since the differences observed in the sympathetic ganglia of the two groups were only quantitative, and also of a minor degree, the results will be considered together.

The investigation centered on the study of the sympathetic ganglia all of which appeared significantly enlarged on macroscopic inspection. No changes were detected in other sectors of the nervous system, including the sensory ganglia The analysis, however, is still in progress and minor differences may have been overlooked at a first inspection. We limit the present report to a description of the major changes observed in the sympathetic ganglia and in the peripheral distribution of sympathetic fibers; a more detailed description will be presented in another paper.[23]

Cell counts and volume measurements were made in the superior and inferior (stellate) ganglia of control and injected mice. Since the microscopic inspection of the para- and prevertebral sympathetic ganglia at all different trunk and head regions revealed the same picture, the results obtained in the superior and inferior cervical ganglia apply to the entire population of sympathetic nerve cells. Therefore we shall speak of volume and cell number increase in the sympathetic ganglia of the injected mice, although only some of these ganglia actually were measured and the cells counted.

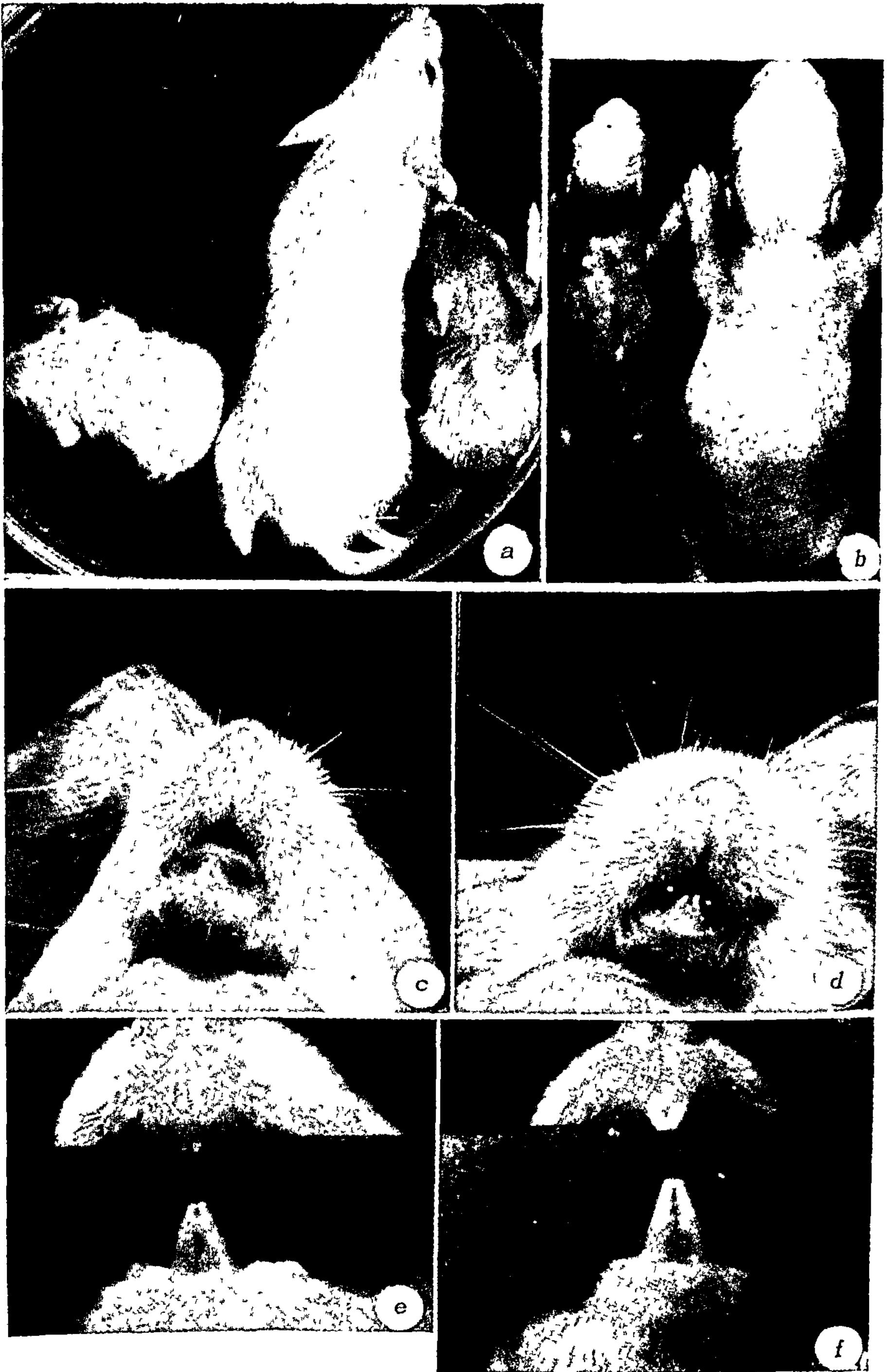


FIGURE 3. Effects on cortisone-injected newborn rats and on newborn mice injected with salivary extract (CM-1 fraction) (*a*) One control and two mice injected with salivary gland extract since day of birth, the darker color of the injected mice is due to lack of hair, control and injected mice 4 weeks old (*b*) Nine-day old rat injected with 0 1 ml of cortisone on day of birth, at the right, an untreated control of same age (*c* and *d*) Lower incisors in two 9-day old mice (*c*) Control (*d*) Mouse injected since birth with salivary gland extract (*e* and *f*) Incisors in control (*e*) and cortisone-injected rat (*f*), the experimental rat received 0 1 ml of cortisone on the fourth and eleventh day, both rats 14 days old. (FIGURE 3*b*, *e*, and *f* reproduced with permission of E J Field and *Journal of Anatomy*[22])

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A twofold increase in the population of the superior cervical ganglia was detected in two mice twelve and nineteen days old, injected since birth with the CM-3 fraction, and compared with controls of the same age. To establish whether such increase was due to an increased mitotic activity (the mitotic activity in the sympathetic ganglia continues until the ninth day after birth) or to a transformation in sympathetic cells of germinal or pluripotential cells present in the ganglia, the mitotic activity of control and injected mice was explored. Mitotic figures in injected and control mice were counted between the second and ninth days. A sharp increase in mitoses was found in all injected mice. The result favors the hypothesis that the increase in number of

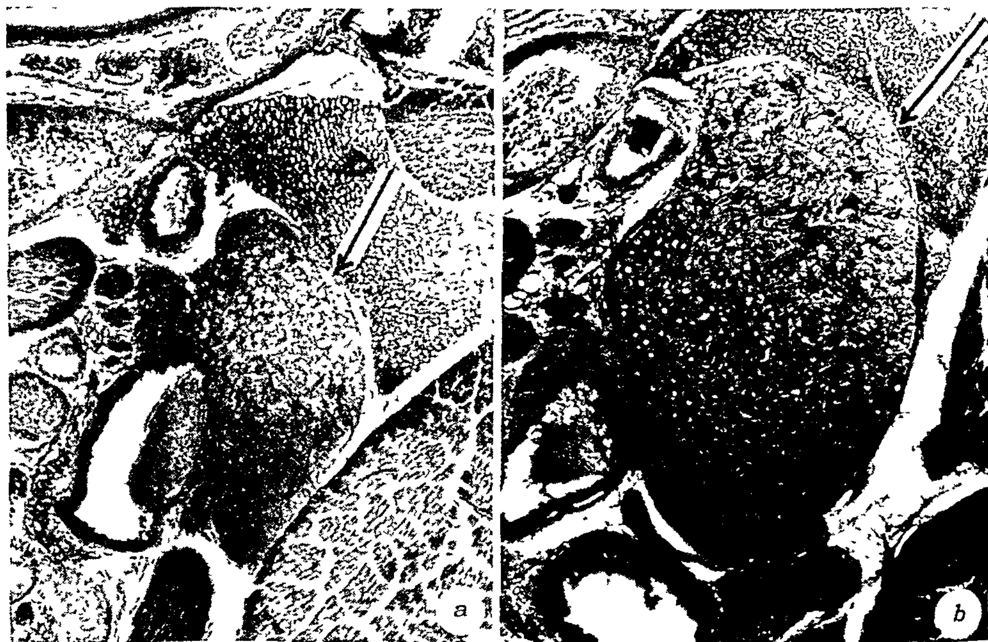


FIGURE 4 Transverse sections through the superior cervical ganglia of mice sacrificed at 9 days; *arrows*, ganglia Silver-impregnated (*b*) Injected since birth with CM-1 fraction.

sympathetic nerve cells is due to an increase in mitotic activity called forth by the salivary factor. Volume measurements of the superior cervical ganglia in 25 experimental and 25 control mice examined between the fifth and twenty-seventh days gave an average increase of the experimental over the control ganglia of 3 to 1 (FIGURE 4). A higher increase was observed in mice injected with the CM-3 fraction and fixed at 19 days. In 2 instances a sixfold increase over the controls was observed in volume of the ganglia of injected mice The over-all increase in volume results from the increase in cell number and to a major extent from the increase in cell size (FIGURES 5 and 6).

The peripheral distribution of sympathetic nerve fibers was not investigated in detail. We can state, however, that the viscera are hyperneurotized and that sympathetic nerves give origin to a much denser fibrillar net around the blood vessels and hair bulbs of injected mice than of control mice.

In a last group of experiments we tested the effect of the CM-1 fraction on

the sympathetic ganglia of adult mice. A total of 50 mice was injected; the sympathetic thoracic chain ganglia were dissected and compared with control chains after 1 to 4 weeks of experimentation. The salivary extract was injected daily in the same proportional doses as used for newborn mice. A

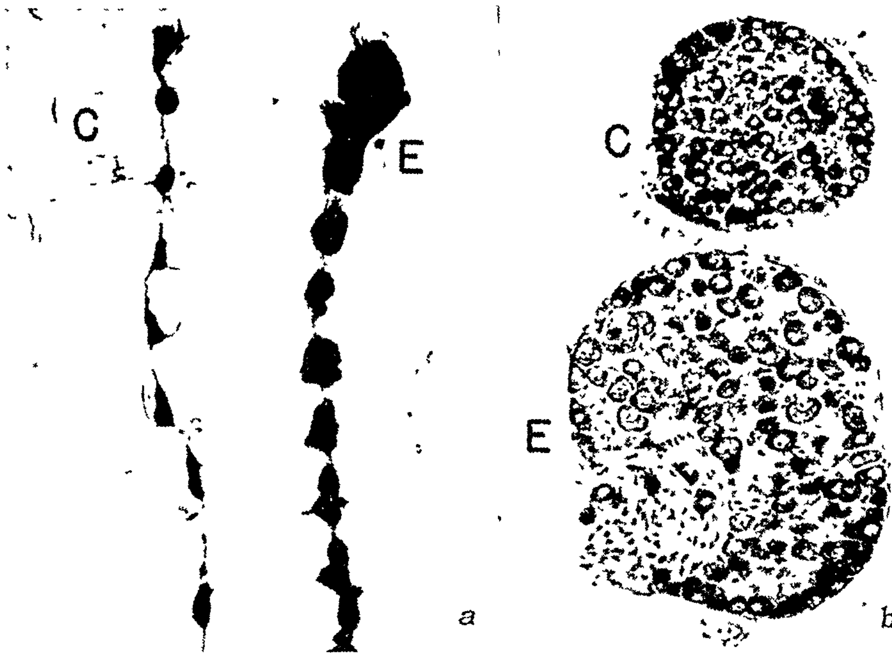


FIGURE 5. (*a*) Whole mounts of sympathetic thoracic chain ganglia of control (*C*) and experimental (*E*) mice 9 days old, *E*, injected since birth with CM-1 fraction of salivary extract. (*b*) Transverse sections through upper (stellate) ganglia of mice of *a*. *C*, control; *E*, experimental

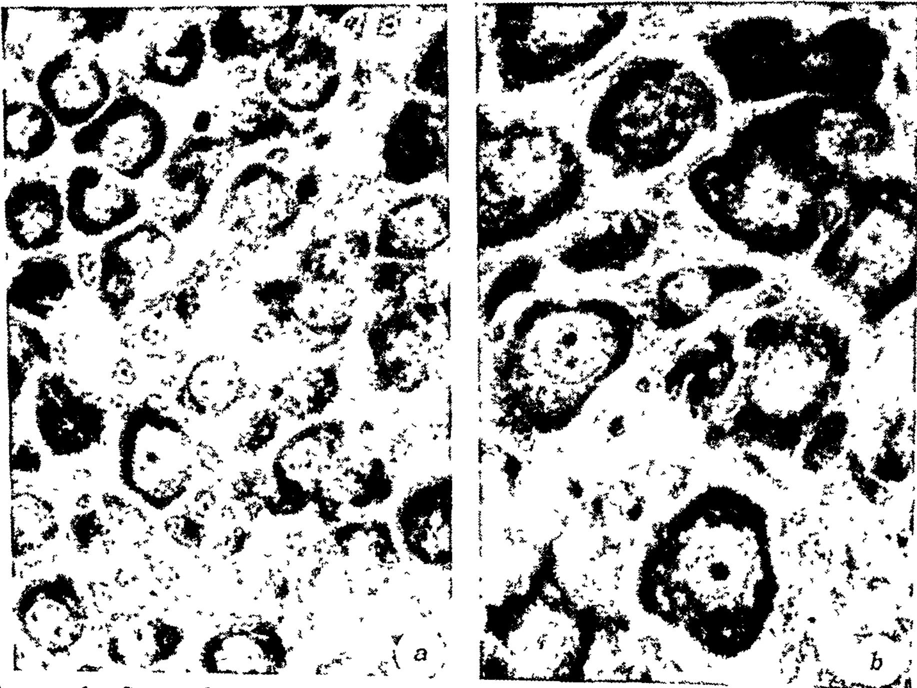


FIGURE 6. Sympathetic nerve cells in superior cervical ganglia of mice sacrificed at 19 days (*a*) Control. (*b*) Injected since birth with CM-3 fraction of salivary factor.

number of ganglia were fixed, stained, and sectioned for histological analysis. An average of a twofold volume increase was observed in the injected mice. No significant differences were found in the number of sympathetic nerve cells in the experimental and control ganglia; cellular hypertrophy was evident in all instances. The over-all increase in the sympathetic ganglia is therefore due to hypertrophy and hyperplasia in the newborn mice, whereas only cellular hypertrophy takes place in adult mice.

Chemical Nature of the Nerve-Growth Factor in the Salivary Extract

The chemical properties of this agent will be reported in another paper[21] Here only some of the results of this investigation will be presented. The agent isolated in the most purified fraction, CM-3, is heat-labile, nondialyzable, destroyed by acid (0.1 *N* NaOH at 26° C.), and stable to alkali treatment and to 6 *N* urea. The ultraviolet absorption spectrum shows a 280/260 absorption ratio of 1 53. Only one peak was seen when the material was examined in a Spinco analytical ultracentrifuge The molecular weight was estimated to be of the order of 44,000 (S.C.). Therefore, it is concluded from this and other evidence to be presented that the salivary factor is a protein, like the mouse sarcomas and the snake venom factors. On detailed examination, however, these do not appear to be identical.

Experiments with an antiserum (*see below*) gave additional evidence in favor of the protein nature of the salivary factor.

Distribution of the Nerve-Growth Factor in Other Mouse Tissues and in Excreta

The *in vitro* experiments mentioned in the first part of this paper gave evidence of a mild nerve growth elicited by embryonic mouse heart Traces of the same activity then were found in homogenates of striated muscle of adult mice and in some instances in the serum of normal mice. Bueker[24] detected activity in partially purified preparations of thymus, kidney, and muscle. We found in some instances nerve-growth activity in the urine of adult mice. Activity in the mouse saliva was also observed. These results deserve some comments The nerve-growth activity detected in other tissues besides the salivary glands and the mouse sarcomas, as well as the activity detected in urine and in mouse saliva, differs in two important respects from the activity of the salivary extract: (1) the activity fluctuates to a considerable extent in preparations from different animals, and (2) even in the best instances the nerve-growth factor is present in a concentration at least 5000 times lower than in the salivary extract The hypothesis suggested itself that the factor is produced in the salivary glands We then proceeded to extirpate the submaxillary and sublingual glands in adult mice Experiments *in vitro* gave evidence of only a mild nerve-growth effect of the parotid glands. These glands were not removed. The operated and control mice were examined one and six months after the extirpation of the glands. Macroscopic and microscopic inspection revealed no appreciable differences between the sympathetic ganglia of operated and control mice. This did not favor the hypothesis that the nerve-growth factor is produced in the salivary glands. The possibility that it is only stored there will be considered in the *Discussion*.

The next experiment was to investigate the *in vitro* effects of an antiserum to the salivary factor. This experiment was expected to shed more light on the chemical nature of the active agent (S.C.). The results did much more than add to our information on the chemical properties of the nerve-growth factor: they revealed that the sympathetic ganglia are not only highly receptive to growth agents, but that they are also vulnerable to antigrowth agents and can be destroyed up to the point of nearly total extinction while other systems are not affected.

Effects of the Antiserum to the Nerve-Growth Factor on the Sympathetic Ganglia of Mammals

An antiserum was prepared by injecting 1 mg. of the purified protein (CM-3) into the foot pads of two rabbits, using Freund's adjuvant. The technique is reported in detail elsewhere.[21] Briefly, it consisted of assaying the effect of the antiserum on the nerve-growth activity of the salivary extract *in vitro*. When it was found that it completely inactivates the nerve-growth effect, the antiserum was tested in newborn mice (S.C.). The experiment consisted of injecting the antiserum in the amount of 0.05 ml./1.5 gm. of body weight in the subcutaneous tissue of newborn mice. The injections were repeated daily for 10 days. The sympathetic ganglia of the treated and control mice were then compared. Upon macroscopic inspection, a striking difference between the 2 pairs of chains was evident. The histological examination revealed an even more impressive picture. Only 7 per cent of the nerve cell population of a control was still present in the superior cervical ganglion of 1 injected mouse. In these, as in the experiments with the nerve-growth factor previously mentioned, the effects observed in 1 ganglion are taken as evidence of the effects of the antiserum on all sympathetic ganglia of the injected animal as verified by inspecting ganglia at different trunk levels. We can therefore say that the injection of the antiserum in this case resulted in the disappearance of 93 per cent of the entire population of sympathetic nerve cells. The surviving cells were highly atrophic whereas satellite cells, although reduced in number, appeared of normal size. These results prompted an extensive and systematic study of the effects of the antiserum in mice injected for different lengths of time and at different stages of development A parallel group of experiments was also performed on newborn rats and rabbits, and on 1 pair of kittens. At first we tested the effect of normal rabbit serum on newborn mice. The serum was injected in the same doses and for the same length of time as the antiserum. When it was found that this serum did not elicit any effect, these experiments were discontinued, and only untreated mice were used as controls.

The injection of the antiserum did not result in any adverse effect on the development of the treated mice, rabbits, or one kitten. A certain percentage of the injected rats developed instead a severe jaundice and died after the first week of treatment. The possibility that the jaundice was due to infection or to a serum disease is now being investigated.

The effects of the antiserum on the sympathetic ganglia of the mice, rats, rabbits, and one kitten were almost identical, however, since a more extensive study was made with mice, this report is based on the results obtained with that species.

The histological examination of 30 injected and 30 control mice sacrificed between the first and twenty-fifth days gave the following results:

(1) The mitotic activity in the injected mice drops sharply even 24 hours after the first injection on the day of birth. At this stage 103 mitotic figures were counted in the superior cervical ganglion of a control mouse, and 6 in the same ganglion of an injected mouse

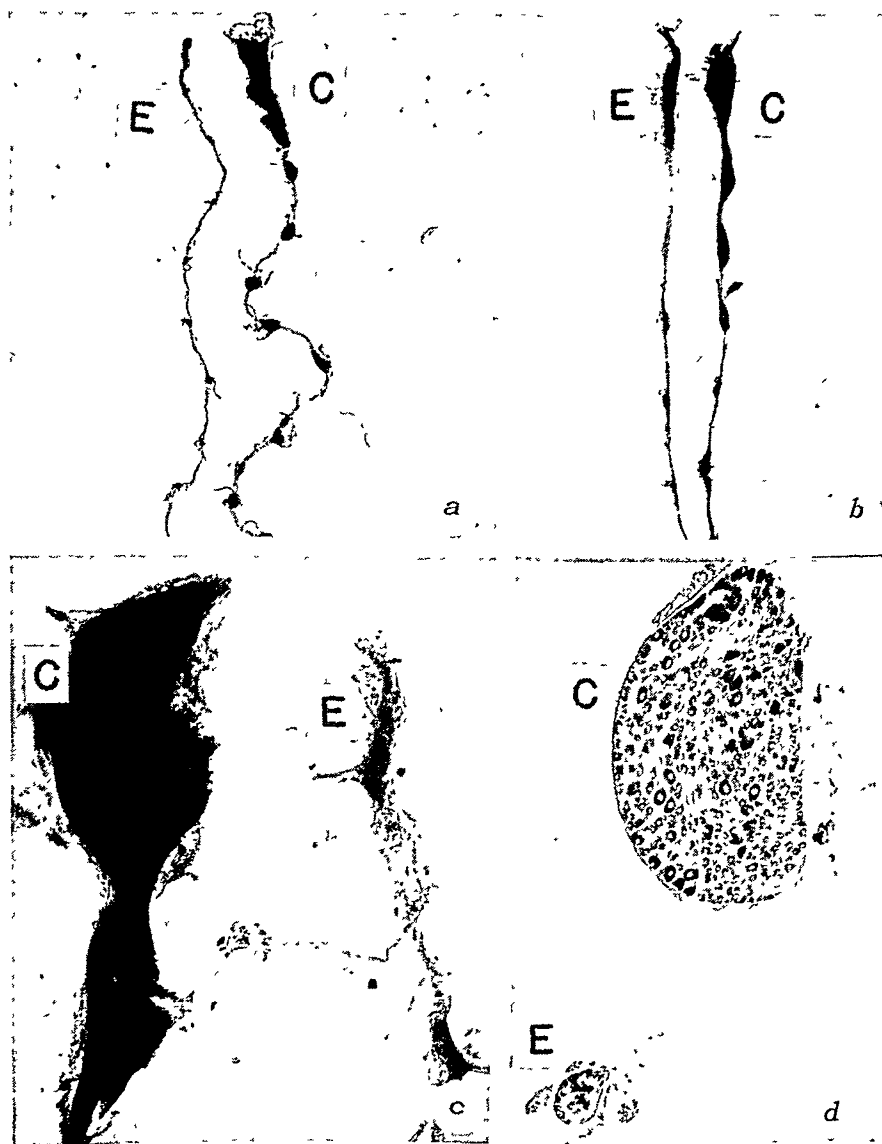


FIGURE 7 (*a*) Whole mounts of sympathetic chain ganglia of control (*C*) and experimental (*E*) mice 25 days old. *E*, injected since day of birth with antiserum to purified salivary factor. (*b*) Whole mounts of sympathetic thoracic chain ganglia of control (*C*) and experimental (*E*) rabbits 4 days old. *E*, injected since day of birth with antiserum to purified salivary factor (*c*) Whole mounts of stellate ganglia of control (*C*) and experimental (*E*) mice three months old, *E*, injected from day of birth to the eighth day with antiserum to purified salivary factor (*d*) Transverse sections through stellate ganglia of mice in *c* *C*, control, *E*, experimental The magnifications are different in the different figures.

(2) The differentiative processes in sympathetic nerve cells are highly affected by the antiserum; the neuroblasts are not only much smaller than those in controls, but the cytoplasm is almost deprived of ribonucleic acid, as shown by the failure to stain with basic dye and the fact that the nucleoli are barely visible.

(3) Dead cells and cell debris are scattered in large numbers among the neuroblasts and satellite cells. The devastating effects of the antiserum become more impressive at a more advanced age. At 25 days only 1 per cent of the nerve cell population is still present in the ganglia. The surviving nerve cells show different degrees of atrophy (FIGURE 7*a* and *b*).

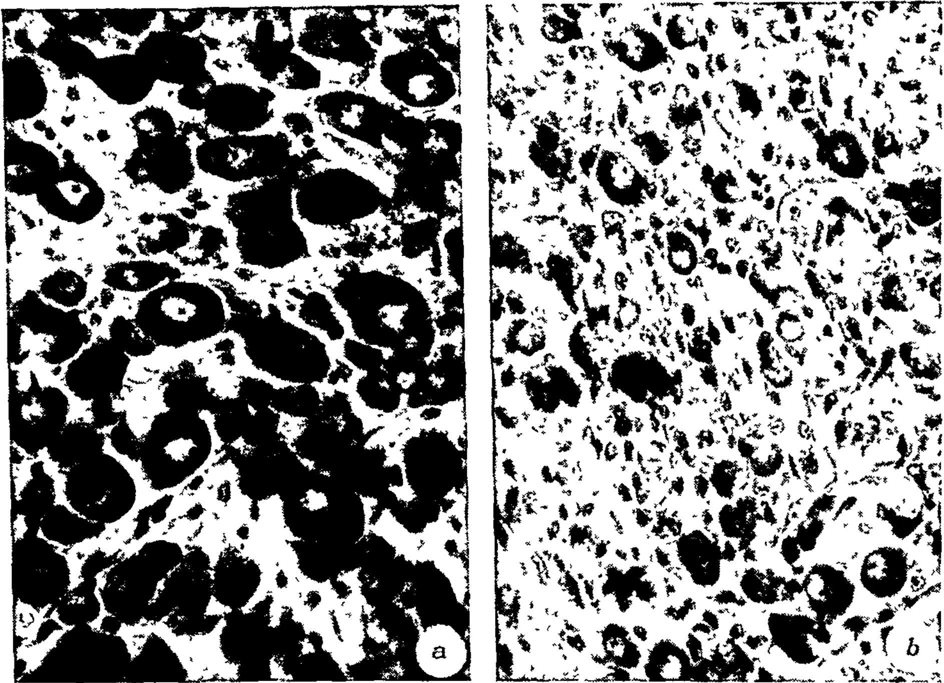


FIGURE 8 Cell density and cell size in superior cervical ganglia of adult male mice. (*a*) Control (*b*) Injected for 20 days with antiserum to purified salivary factor

It was of interest to establish (1) the time necessary to obtain by daily injections the total or nearly total destruction of the sympathetic ganglia, and (2) the effects of the antiserum in mice sacrificed many months after discontinuance of the treatment. Both problems are still under investigation. Preliminary results indicate that the treatment of newborn mice with antiserum for a period of 1 week results in the nearly total destruction of the sympathetic nerve cells. Cell counts made 2 months after discontinuance of the treatment showed a residual nerve cell population of about 1 per cent of that of the controls (FIGURE 7*c* and *d*). A more detailed report of these results is given in another paper.[25]

In another group of experiments also in progress we are testing the effects of the antiserum in adult mice Daily injections for 20 days resulted in a reduction of the population of sympathetic nerve cells to 30 per cent of that of the controls (FIGURE 8). The effects therefore appear to be similar, although

less severe than in newborn mice. It remains to be seen if injections protracted for a longer time or with a more potent antiserum will result in the nearly total destruction of sympathetic nerve cells in adult animals as well.

The injected newborn and adult mice showed no adverse effects on other systems. The development of young mice with only 1 per cent of the population of sympathetic nerve cells still present did not differ from that of the control mice.

Discussion

Before attempting a discussion of the observed effects, a short summary of the chief results of our investigation seems desirable.

(1) Potent nerve-growth factors were isolated from mouse sarcomas, snake venoms, and mouse submaxillary salivary glands.

(2) The chemical properties of all these factors indicate that they are proteins, but probably *not* identical.

(3) The biological activities of the three factors are remarkably similar, if one may judge from their *in vivo* and *in vitro* effects. They all enhance the production of sensory and sympathetic nerve fibers from ganglia explanted *in vitro* from the chick embryo. More extensive experiments with the salivary factor proved that it also has the same effect on ganglia of mice, rats, and human fetuses. Experiments *in vivo* consisted of transplantation of fragments of mouse tumor in the chick embryo, or of the injection of snake venom or the salivary extract into the yolk of developing chick embryos. In all instances we observed an overgrowth of the sympathetic ganglia and a hyperneurotization of the viscera. The salivary factor elicited also an overgrowth of the sensory ganglia. This last effect was observed in intraembryonic transplantations of mouse tumor; it was less apparent with snake venom.

(4) The salivary factor, the last isolated and the most potent of the three factors, was also assayed on newborn and adult mice. In newborn mice it caused in the best cases a volume increase in the sympathetic ganglia that was six times the volume of control ganglia. The over-all increase is due to an increase in cell number and in cell size. Hyperneurotization of the viscera and blood vessels was observed in all injected animals. No effects were detected in other sectors of the nervous system or in other organs. The animals injected with the most purified salivary factor appeared as healthy and vigorous as the controls

(5) The injection of the partially purified salivary extract in newborn mice has other effects besides those on the sympathetic ganglia. These effects (dwarfism, failure of hair to grow, precocious opening of the lids, and precocious eruption and calcification of the upper and lower incisors) are remarkably similar to the effects of cortisone on newborn rats.

(6) While the extirpation of the salivary glands in adult mice did not affect the sympathetic ganglia, the injection of an antiserum to the salivary protein in newborn mammals resulted in drastic effects on these ganglia. The sympathetic ganglia in newborn mice, rats, rabbits, and one kitten showed, in fact, a very high degree of atrophy. In mice only 1 per cent of the nerve-cell population was still present after daily injections for a period of 8 to 20 days. The

atrophy of the sympathetic ganglia in adult mice was less severe, but similar to that observed in newborn mice.

The results summarized above shed light on some of the basic problems of growth, differentiation, and maintenance in the sympathetic ganglia of mammals. They indicate, in fact, that some nerve-growth factors manufactured in animal tissues can call forth a sixfold increase in the sympathetic ganglia and cause the hyperneurotization of the viscera without affecting in any appreciable way the function of the hyperneurotized organs and, in fact, of the entire organism. They also showed that 99 per cent of the population of sympathetic nerve cells of mice can be destroyed with no effects observed under laboratory conditions

These findings are of interest not only in connection with the sympathetic nervous system, but also from a more general biological point of view. One may, in fact, wonder whether other specific growth agents are produced in the organism and are equally essential to the growth and maintenance of other systems.

It is particularly pertinent to the main topic of this monograph to consider some questions raised by these results. What is the significance of a nerve-growth factor in the mouse submaxillary salivary glands? What is the significance of the cortisonelike effects evoked by this extract, and what is the functional meaning of the linkage between the salivary gland and the sex hormones and other hormones, as described by many authors? Are the cortisonelike factor and the nerve growth factor produced in the salivary gland? We are not in a position to answer these questions, but some considerations of the above results may indicate a possible line of approach to the problem. The last question seems to offer the best chances of finding an answer and this in turn could well result in an answer to other questions.

Two alternative hypotheses were suggested. Either the nerve-growth factor and the cortisonelike factor are produced, or they are simply stored and released, from the salivary glands. The following arguments seem to favor the second alternative.

(1) We have evidence that mouse sarcomas manufacture the nerve-growth agent even when transplanted in the foreign environment of the chick embryo. Such evidence thus far has not been produced by experiments with salivary glands.

(2) The extirpation of the salivary glands of mice did not result in any appreciable effect on their sympathetic systems. We should have anticipated this result if the salivary glands were the only, or at least the most potent, source of this agent harbored in such a large amount in the gland.

(3) The nerve-growth agent was found in high concentration in the snake venom, it was also detected in mouse saliva. These results indicate that the growth factor is released through the exocrine channels of the salivary gland or of its homologue, the venom gland. Since these factors lose their activity when they are exposed to proteolytic enzymes, their presence in the snake venom and in the mouse saliva seems to be of no functional value.

(4) The cortisonelike effect of the less purified salivary extract poses the question of the production of this agent. Since it replicates the performance

of another well-identified agency, the adrenal gland, the most plausible explanation is that it is produced in the adrenal and only stored in the salivary gland. The possibility of its supplementary production in the salivary glands seems very remote.

The varied and complex functions of the salivary glands have been ignored or at least underrated for a long time; we would, however, take the risk of overrating these functions now by assigning to the salivary glands many different and vital tasks, such as the production of nerve-growth factors and of cortisone-like hormones.

We therefore propose to consider the other alternative: The gland may store in its spacious tubular portion a number of different agents, and it may dispose of them through its excretory channels or release them in the circulation as the need may arise. This hypothesis is being tested in investigations now in progress.

It seems justifiable to hope that the nerve-growth factor isolated from mouse salivary glands may eventually shed light on some functions of the salivary glands. If so, we should have repaid the salivary glands our debt of gratitude for the light they shed on the sympathetic nervous system.

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EFFECTS OF RADIATION UPON THE SALIVARY GLANDS

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Introduction

The nature of this publication is such that it appears desirable to relate radiation effects upon salivary glands to general considerations of metabolism rather than to emphasize technical aspects of radiobiology that would be of interest to specialists in that field. The technical data that I present are therefore reduced to the minimum that will illustrate radiobiological applications that may be of interest. One aspect of the general problem is the difficulty of relating structure to function at microscopic and submicroscopic levels. This problem also exists in radiobiology, and several recent investigations that suggest solutions will be discussed.

The application of ionizing radiation to living tissues produces a traumatic reaction that is not unlike those observed when large doses of sunburn (ultraviolet) or heat (infrared) are applied. In actual experimentation it has been found that denaturization of protein by heat is accomplished more readily after the protein has been exposed to X radiation and, conversely, that the sensitivity of certain enzymes to ionizing radiation is greater at higher than lower temperatures.[1] Thus there is evidence that irradiation produces effects similar to those of heat denaturation. From an experimental point of view there are certain advantages in using ionizing radiation (for example, X rays) as a traumatic agent, rather than applying trauma or stress of various other kinds the amount of radiation that is absorbed by the tissues can be measured in specific energy units, the radiation can be directed at the whole experimental animal or at only a few cells of a given tissue, and the exposure can be continuous or intermittent. Thus, ionizing radiation becomes a tool of considerable accuracy, and the number of ionizations that would be produced per molecule of protein within a cell exposed to 1000 r can be calculated

The effects of radiation vary with the cell, the organ, and the type of animal exposed. The reason that rabbits can tolerate a total body dose of X radiation of approximately twice the magnitude that is lethal to dogs is not well understood, but it is probably a reflection of variable sensitivity of specific cells within the animals This is begging the question, however, for the reason for the variation in sensitivity among cells is not entirely clear. The search for reasons for this variability leads eventually to the molecular level. In the studies discussed in this paper, the effects of ionizing radiation on protein molecules that function as enzymes are analyzed in relation to the microscopic structural changes observed in the tissues of origin.

Physiological and Morphologic Effects of Radiation

When X rays are used to treat pathological lesions about the face, it is noted that there are functional changes in the salivary glands. The saliva may at first flow copiously, but soon there is a marked reduction in flow of highly viscid

saliva and eventually, with sufficient irradiation, there may be complete stoppage. Experimentation with animals[2-4] has shown that, although fibrotic changes are observed several months after exposure of salivary glands to doses of X ray in the 1500 r range, limited changes occur immediately following exposure, as illustrated by changes observed in the salivary glands of rats.[3]

The specific salivary gland of the rat that consistently exhibited histological changes following radiation was the submaxillary gland. This is a paired gland lying near the median line, just above the hyoid bone. It is the largest single mass of salivary tissue and, together with the greater sublingual gland, it forms a capsulated almond-shaped gland. The submaxillary gland contains three easily identifiable structures: acini, tubules, and collecting ducts. The acini are smaller than the tubules and consist of irregular masses of cells that are roughly pyramidal in form. The acinar cells contain round or ovoid nuclei located basally and measuring about 10 μ in diameter. The nuclei in which chromatin is distributed almost evenly are darkly basophilic and usually contain one nucleolus. The cytoplasm in fixed preparations is somewhat irregularly distributed, faintly basophilic with an acidophilic background. The tubules consist of cuboidal or low columnar cells with nearly centrally placed nuclei of good size (about 15 μ). A definite central open space exists within the tubule. The collecting ducts are similar to the tubules in staining characteristics, the main differences being that the ducts are larger and have a larger central open space, the cells are tall columnar with vertical striations basally, and no mucinogen granules are produced.

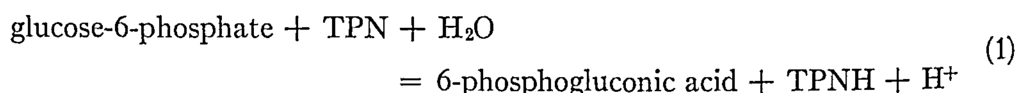
The radiation changes observed in the submaxillary gland were sufficient to be noted at relatively low magnification ($\times$100) and consisted of variability in size and staining characteristics of the nuclei of the acinar cells. A detailed examination at $\times$200 and $\times$475 magnification revealed extensive nuclear pleomorphism and a variation in nuclear size of from 5 to 30 μ. Many of the nuclei that were abnormal in size also took a heavy basic stain in hematoxylin and eosin, which emphasized size variability. The peculiarly shaped nuclei included stellate and discoid forms, which frequently stained heavily with hematoxylin and were thus very noticeable. However, the three nuclear changes described did not always occur simultaneously. Size of whole cells was not easily discernible, for the cellular membranes were indistinct. Frequently, however, when the nucleus of an alveolar cell was large, there was an equal increase in cytoplasm about the nucleus, and these "giant" cells were optically prominent. Changes in the tubules and ducts of this gland were not found to be consistent enough to be reliable indicators of radiation damage. Often one had the impression that architectural disarrangement or actual epithelial dislodgment of cells was a characteristic of the exposed gland, but the same condition was sometimes found in control animals.

Biochemical Effects of Radiation

The described morphologic changes were consistently found in irradiated salivary glands of rats examined 16 or more days after exposure; during the first five days, however, no morphologic changes were observed. Since physiological changes appear within a few hours after radiation, it was of interest to

study the metabolic activities of salivary glands after radiation and to learn whether submicroscopic changes could be measured with the use of biochemical techniques. The method selected for evaluating the metabolic activity of salivary glands was the determination of the individual activities of enzymes occurring in established pathways of metabolism. Two of the enzymes studied, which will be considered in this paper, are glucose-6-phosphate dehydrogenase (G-6-PD) and isocitric dehydrogenase (ID).

The basic reactions, shown below, may be followed by observing changes in the optical density of pyridine nucleotides, which are biologically required for reduction or oxidation.



$$\text{glucose-6-phosphate} + \text{TPN} + H_2O = \text{6-phosphogluconic acid} + \text{TPNH} + H^+ \quad (1)$$



$$d\text{-isocitrate} + \text{TPN}^+ = \text{oxalosuccinate} + \text{TPNH} + H^+ \quad (2)$$

In each of the reactions a protein catalyst is required and is known as the dehydrogenase of the corresponding substrate. Since the reaction rates are proportional, within certain limits, to the concentration of the required enzymes, it is possible to estimate the enzyme concentration in each case by determining the rate of reduction of the respective pyridine nucleotide. The establishment of an arbitrary enzyme unit permits comparisons to be made among the enzyme activities of the systems under study. One enzyme unit is defined as the amount of enzyme that will cause an increase in optical density of 0.01 at wave length 340 mμ during a half-minute period early in the reaction.

In the first series of experiments,[5] the salivary glands of the rats were exposed to 3000 to 5000 r of X ray through a 24- or 32-mm. aperture in such a way that all of the animal was shielded except the cylinder of tissue that included the salivary glands This region, of course, included other structures in the region of the salivary glands, such as parts of the esophagus and trachea. At periodic intervals following exposure, assays were performed on supernatant fractions of centrifuged homogenates of the submaxillary-sublingual salivary glands, and similar assays were done on unirradiated control glands.

Before the results of the enzyme assays are given, it should be mentioned that certain general effects resulting from the local radiation of the rats were reflected in the measurements of enzyme activity. A change in body weight, salivary gland weight, and extractable protein content of the salivary glands occurred between the time of radiation and killing. A maximum total weight loss of 35 to 40 per cent of the original weight of the animals was observed between the seventh and twelfth days.

A loss in salivary gland weights, roughly paralleling the loss of total body weight, amounted to as much as 50 per cent for the radiated glands when compared to average weights of nonradiated glands, except in the case of animals sacrificed immediately after exposure

Assays for G-6-PD in these experiments showed that there was a significant rise in specific activity following radiation that continued as long as twenty days following exposure. However, since specific activity is related to protein

content and since it was observed that there were weight changes in the salivary glands, calculations were made as to the activity per total paired salivary glands. When this was done, the activities of radiated glands fell within the range of nonradiated glands. The values given in the following table are expressions of the total enzymatic rate less the endogenous rate for the control (C) and radiated (R) salivary glands in two separate experiments:

Specific Activity, G-6-PD	Activity per Pair of Glands, G-6-PD
C 1 12 ± 0 27	C 3 92 ± 1.16
C 1 26 ± 0 48	C 5.09 ± 0 75
R 2 17 ± 0 58	R 4 2 ± 0 92
R 2 72 ± 0 73	R 4 3 ± 0.66

The values for specific G-6-PD activity are significantly different for the control and radiated groups, with a probability of 0.001. The activities compared on the basis of whole paired salivary glands are shown to be not significantly different when analyzed by the Student *t* test.

The isocitric dehydrogenase activity of control salivary glands of rats is 3 or 4 times as great as G-6-PD activity when measured in the same way. Following radiation, the specific ID activity rises significantly above the control value within 1 day and remains there from 13 to 20 days However, when the ID activities are calculated for whole paired glands, there is found to be a decrease in activity following radiation, which becomes significant in 3 to 8 days. The values, expressed in total less endogenous rates for C and R rats in two specific experiments are as follows.

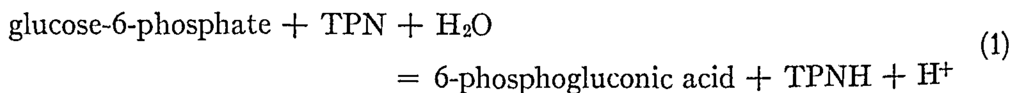
Specific Activity, ID	Activity per Paired Glands, ID
C 3 95 ± 0 52	C 13 6 ± 1.73
C 3 68 ± 1 14	C 14 4 ± 1 94
R 5 62 ± 1 40	R 10 4 ± 2 44
R 6 56 ± 1.15	R 10 6 ± 2.22

The values for specific activity differ significantly for the C and R groups, with a probability value of 0.001 The ID activities for paired glands showed significant differences for C and R rats, with probability values of 0.01.

Since the rates of enzyme action for G-6-PD and ID seem to vary in significance for C and R animals, depending on whether the activities were related to wet weight, protein content, or total activity per paired glands, another experiment was undertaken[6] in which the weight changes of rats and of salivary glands might be avoided. In this study there was an attempt to protect all of the rat from radiation except the submaxillary-sublingual salivary gland on one side of the animal. This was accomplished surgically by exteriorizing the left salivary gland, placing a small shield beneath it and, at the same time, protecting the rest of the animal as in the previous study. This can be done without cutting the vascular or nerve supply to the glands. The salivary glands from the shielded side of the rats were evaluated for enzyme activity simultaneously with the radiated glands, and this report gives a comparison of the values obtained. An additional series of rats was carried through the surgical procedures

study the metabolic activities of salivary glands after radiation and to learn whether submicroscopic changes could be measured with the use of biochemical techniques. The method selected for evaluating the metabolic activity of salivary glands was the determination of the individual activities of enzymes occurring in established pathways of metabolism. Two of the enzymes studied, which will be considered in this paper, are glucose-6-phosphate dehydrogenase (G-6-PD) and isocitric dehydrogenase (ID).

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content and since it was observed that there were weight changes in the salivary glands, calculations were made as to the activity per total·paired salivary glands. When this was done, the activities of radiated glands fell within the range of nonradiated glands. The values given in the following table are expressions of the total enzymatic rate less the endogenous rate for the control (C) and radiated (R) salivary glands in two separate experiments:

Specific Activity, G-6-PD	Activity per Pair of Glands, G-6-PD
C 1.12 ± 0 27	C 3.92 ± 1 16
C 1 26 ± 0.48	C 5 09 ± 0.75
R 2.17 ± 0 58	R 4.2 ± 0 92
R 2.72 ± 0 73	R 4 3 ± 0.66

The values for specific G-6-PD activity are significantly different for the control and radiated groups, with a probability of 0.001. The activities compared on the basis of whole paired salivary glands are shown to be not significantly different when analyzed by the Student *t* test.

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Specific Activity, ID	Activity per Paired Glands, ID
C 3 95 ± 0 52	C 13 6 ± 1 73
C 3 68 ± 1.14	C 14 4 ± 1.94
R 5 62 ± 1 40	R 10 4 ± 2.44
R 6.56 ± 1 15	R 10 6 ± 2.22

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alone and evaluated for enzyme activity. The enzyme activity rates in arbitrary units are as follows:

G-6-PD Activity

Control	3 3 ± 0 71	p = 0 05
Radiated	3.8 ± 0 67	
Second week only:		
Control	3.04 ± 0 62	p = 0 01
Radiated	3 75 ± 0 68	
Surgical control.		
Surgical side	3 9 ± 0 2	
Unoperated	3.7 ± 0.4	

ID Activity

Control	9 3 ± 1 6
Radiated	9 3 ± 1 8
Surgical control	
Surgical side	10.5 ± 0 9
Unoperated	10 1 ± 0 3

Insofar as could be determined objectively, the rats were not affected adversely by either the surgical procedures or the radiation. The gauze covering the treatment area spontaneously came off in 3 or 4 days and all wounds healed by first intention. Animals consumed normal amounts of food and water after treatment, and there were no marked changes in body weight. A comparison of the average salivary gland weights of radiated and nonradiated glands showed that there was a loss of weight after radiation, but this was not found to be significant when evaluated by the Student *t* test. The values for glucose-6-phosphate dehydrogenase activity were higher for radiated salivary glands than for nonradiated glands in all groups of rats except one. An evaluation of the increase in enzyme activity for the whole series of animals by the Student *t* test showed it was significant, with a probability of 0 05. A similar analysis of enzyme activity values for animals sacrificed from the eighth through the fifteenth day following exposure showed a higher significance (p = 0.01).

The mean values for ID activity were essentially the same for R and C salivary glands. It is also noteworthy that the mean nitrogen values were essentially the same for R and C tissues and that the calculated values for protein were 1.22 and 1 25, respectively, for the amounts of solution used in each test (0.2 ml).

Discussion

The submaxillary glands are not homogeneous structures, but are made up of secreting acini, tubules that also have some secretory function, collecting ducts, and supporting connective tissue It is well known that different cells may differ in sensitivity to radiation by many orders of magnitude; therefore, depending upon the location of the enzymes in specific cells, variable effects of radiation upon the enzyme activity might be expected. It is possible to make a crude separation of the parenchyma of glandular tissue from its fibrous connective tissue stroma by forcing the softer parenchymatous material through small holes of a sieve. In early experiments with C animals such separations were made, and assays for G-6-PD and ID showed that the activities in parenchymal cells were relatively higher for ID and lower for G-6-PD In the last series of experiments, in which the salivary glands were exteriorized for irradiation, there was an increase in G-6-PD activity during the first week after exposure and a more significant increase in the second week. This suggests that the reparative processes taking place in the connective tissue elements of the gland required accelerated metabolism to supply them with energy and building

blocks for repair. There is also the possibility that one metabolic pathway, such as the citric acid cycle, is predominant in the secretory functioning of the gland, while another, such as the shunt pathway, is more involved in anabolic processes However, no change in ID activity was observed as the result of radiation in the last experiment, even though physiologically radiation does effect salivation. Of course, another enzyme in the citric acid cycle could have been affected, and the total rate of metabolism by this pathway would be reduced to the rate of the limiting enzyme component.

The first two series of experiments emphasize the importance of relating enzyme activity (that is, "specific enzyme activity") to more than just the protein content of the tissues. Distinctly different interpretations of the data are possible, depending upon whether the enzymatic activity per unit of wet-weight tissue, per unit of protein, or per paired salivary glands is considered. In the case of ID, it was observed in the first experiment that the specific enzyme activity increased significantly with irradiation while the enzyme activity of the total organ remained unchanged. This could be due to a variety of factors, such as loss of specific kinds of protein, selective damage of certain kinds of cells, or an indirect effect caused by destruction of an enzyme inhibitor. Similar effects of radiation on spleen and intestine have been observed by Feinstein,[7] who points out that the data show differences that are only mathematical artifacts due to changes in organ weight or loss of protein. Feinstein also points out the heterogeneous character of these organs and the possibility of variable sensitivity to irradiation among the cell populations.

A somewhat different point of view is taken by Smith and Low-Beer,[8] who review the findings of other investigators along with their own, relative to changes of enzyme activity following radiation, depending on whether the activity is related to the mass of the total organ or to the protein content of standard aliquots. These investigators believe that such data give evidence as to the location of the enzymes in certain cells of the organ or show whether they are distributed equally throughout the organ. They feel that an extension of results of this kind to include many enzyme systems, combined with concurrent histological studies, could further our knowledge regarding the mobility of cell populations following radiation, and perhaps could elucidate biochemical distinctions between cell types.

Finally, the advantages of using the salivary glands on one side of the animal for experimentation and the salivary glands on the opposite side as the controls should be emphasized Even though no general effects of radiation were observed in the series of experiments when this was done, there could have been subclinical effects. However, any such undisclosed general effects should have been elicited in the salivary glands on both sides of the animals, therefore, the differences in enzyme activity between the right and left sides should represent the local effects of radiation. This is particularly true since the surgical control glands did not differ in enzyme activity from the unoperated paired salivary glands. Since there was a difference in the G-6-PD activity and no difference in the weights of the glands or nitrogen values for comparable aliquots taken from radiated and control glands, we can conclude that both the specific and organ enzyme activities were increased. This increase in G-6-PD would

appear to be related to the functioning of the gland, for example, to its repair, since its significance was only borderline during the first week and at the 1 per cent level during the second week. Increased production of G-6-PD was apparently induced by the radiation damage. Such an increase could be a general reaction to trauma and not specific to radiation alone.

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GLYCOGEN CONTENT OF HUMAN NORMAL BUCCAL MUCOSA AND BUCCAL LEUKOPLAKIA*

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In the present study the glycogen concentration of excised human buccal mucosa was compared with the glycogen content of buccal leukoplakia. For the normal mucosa, samples from the buccal area were removed from 10 subjects in the age group of 40 to 55 years. These subjects were completely free of leukoplakia.

In another group of 10 subjects, 3 different buccal mucosal specimens were excised from the same patient and were analyzed for glycogen content. The 3 different specimens were leukoplakia, normal mucosa adjacent to leukoplakia, and normal mucosa at a considerable distance from the leukoplakia.

Methods

Samples of buccal mucosa were excised under local anesthesia The samples varied in weight from 50 to 80 mg.

The glycogen content of the tissue was determined by the method of Walaas and Walaas.[1] Because the tissue of buccal mucosa contains an appreciable amount of fat, it was found necessary to wash the glycogen precipitate with an alcohol-chloroform mixture before further purification. After purification, the glycogen was hydrolyzed to glucose, and the glucose formed was determined by the method of Nelson [2]

Results

The content of glycogen in specimens of human buccal mucosa removed from normal mouths is five times greater than the content of glycogen found in buccal leukoplakia Normal-appearing mucosa adjacent to leukoplakia contains only one half of the glycogen content found in normal mucosa from normal mouths, while specimens of normal-appearing mucosa removed from areas distant from the leukoplakia contain only two thirds as much glycogen as is found in normal mucosa from normal mouths. See TABLES 1 and 2.

Summary and Conclusions

The glycogen concentration of human buccal mucosa from normal mouths was determined and compared with the glycogen concentration of leukoplakia and of normal-appearing mucosa from mouths containing leukoplakia. The glycogen concentration diminished in the order of buccal mucosa from normal mouths to leukoplakia.

These findings suggest that diminished glycogen concentration may be a biochemical change that precedes the morphologic changes found in leukoplakia.

* The work described in this paper was supported in part by a grant from the National Research Council, Washington, D. C

TABLE 1
GLYCOGEN CONTENT OF NORMAL HUMAN BUCCAL MUCOSA
Normal Mucosa from Mouths Free of Leukoplakia

Number of individuals	μM per gm /wet weight	Range μM/gm
10	32.70	19 32 – 51 90

TABLE 2
GLYCOGEN CONTENT OF BUCCAL LEUKOPLAKIA AND NORMAL BUCCAL MUCOSA FROM THE SAME PATIENT

	Number of individuals	μM/gm wet weight	Range μM/gm
Samples of leukoplakia	10	6 44	3 71 – 12 40
Clinically normal samples adjacent to leukoplakia	10	15 54	14 72 – 16 10
Clinically normal samples distant from leukoplakia	10	20 01	12 87 – 26 10

Studies on glycogenesis in human buccal mucosa have been completed and will be reported in the near future.

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ELECTRON MICROSCOPE STUDIES OF KERATINIZATION AS OBSERVED IN GINGIVA AND CHEEK MUCOSA*

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Knowledge of the microstructure of normal oral soft tissue is of fundamental importance to a better understanding of the metabolism and pathological disturbances to which the mouth is prone. Very little has been reported on electron microscopy of oral epithelial tissues outside of three previous publications from our laboratory (Sognnaes and Albright, 1956; Sognnaes *et al.*, 1956; and Sognnaes and Albright, 1958). However, during the past five years there have appeared more than a dozen publications on electron microscopy of skin and its derivatives, notably hair. Special reference should be made to the studies by Selby (1955); Birbeck and Mercer (1957); Odland (1958); Horstmann and Knoop (1958), Hibbs and Clark (1959); and Charles (1959).

Skin and oral tissues present many comparable structural features, as well as similar technical difficulties, in contrast to the more homogeneous soft structures with which most electron microscopists have hitherto been primarily concerned. One of the basic processes of squamous epithelial cells is keratinization, a subject of many controversial observations. The present paper will attempt, first, to describe certain normal epithelial structures of human gingiva and other parts of the oral mucosa as seen in the electron microscope, and, second, to discuss the possible relations which some of these structures may have to those in skin in general and to the process of keratinization in particular. Special attention will be directed to the so-called tonofibrils, intercellular bridges, and keratohyalin granules

Materials and Methods

The following observations were based on examination of small pieces of normal human gingiva and of mucosa from the human and rat cheek.

Specimens were fixed from ½ to 4 hours in 1 per cent OsO_4 buffered with Veronal acetate to *p*H 7 5, according to the procedure of Palade (1953); then they were briefly washed in distilled water, dehydrated in increasing concentrations of ethyl alcohol, and embedded in a 4:1 mixture of *n*-butyl methacrylate and methyl methacrylate. Capsules were left in an oven overnight at 55° C. to polymerize.

Blocks were cut with a Porter-Blum microtome. Both glass knives and diamond knives were used. Sections were examined without removing the plastic by the use of an RCA model EMU-2B electron microscope with a 40-mm. objective operature. Electron micrographs were made at original magnifications of 2000 to 8000 and enlarged photographically from 2½ to 7 times.

Observations

In the deep Malpighian basal layer of the oral epithelial cells the nuclei were large and rounded with prominent nucleoli, which exhibited a typical coiled

* This study was supported in part by Grant D-662 (C-1) from the National Institute of Dental Research, Public Health Service, Bethesda, Md.

structure, whereas the cytoplasm was made up of small particulate matter interspersed with small fibrils and numerous mitochondria. The vesicular type of endoplasmic reticulum seen in many other types of cells was not discernible (FIGURE 1). As noted in similar sections of human skin, the mitochondria failed to exhibit a definite internal structure even though the surrounding organelles seemed to be reasonably well fixed.

One of the outstanding features of the upper stratum spinosum cells of the

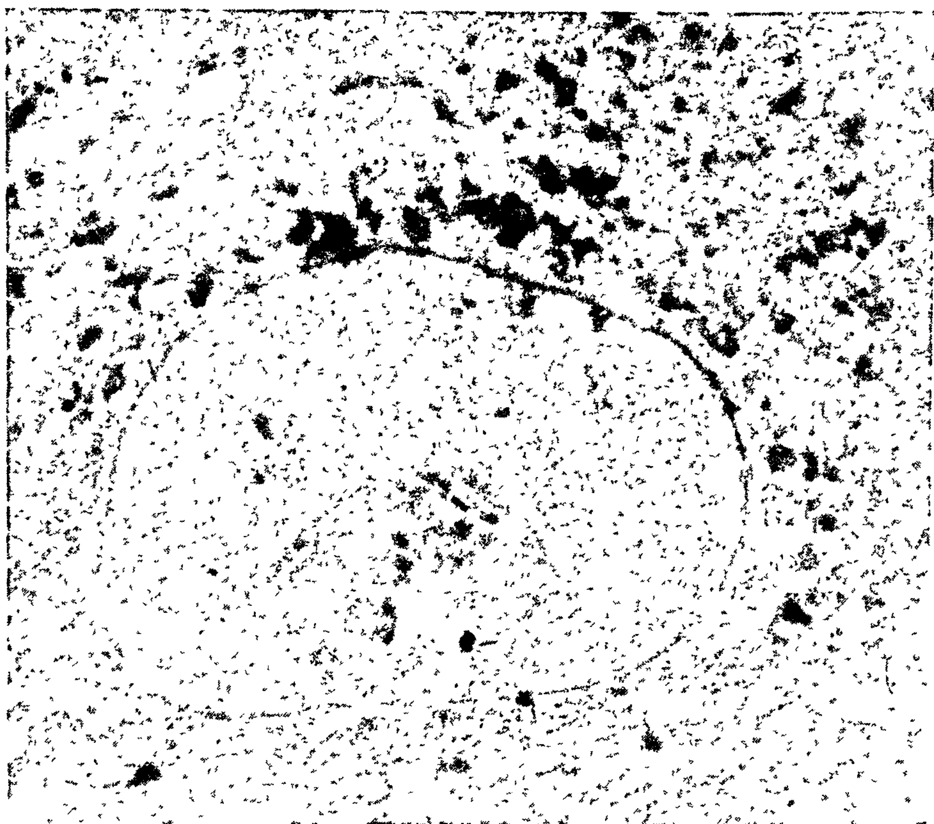


FIGURE 1 Epithelial cell from the lower part of the stratum spinosum of the human gingiva The endoplasmic reticulum is finely granular rather than vesicular The mitochondria are plentiful, the nucleus is large and well rounded, the nucleolus is prominent and has a coiled structure ×11,500

gingiva was the prevalence of tonofibrils. These were also seen in other parts of the oral mucous membrane, but were not as elongated or as well organized. In fact, the gingival tonofibrils were so abundant that other cytoplasmic organelles seemed to be pushed aside and either confined along the nuclear membrane or outer cell wall (FIGURE 2) Another characteristic of these gingival epithelial cells was the extension of long tonofibrils into the fingerlike projections of the outer cell wall, possibly forming an intracellular "attachment" to the desmosomes or nodes of Bizzozero (FIGURE 3) On the other hand, there did not appear to be a direct continuity of cytoplasm or tonofibrils from one cell to another, that is to say, no intercellular attachment *between* the cells

The so-called intercellular bridges, when seen at the ultrastructural level of

observation, represented zones of intimate juxtaposition rather than organic fibrillar union. Where there existed a slight cell shrinkage, as noted in the gingival prickle cells of FIGURE 3, the attachment plaque appeared to be more firm than simple adhesion as previously suggested by Weiss (1958). An enlarged view of the desmosomes as seen in FIGURE 4, a specimen from the cheek mucosa of the mouse, shows a distance between the two contact plaques of about 300 Å. Delicate tonofilaments run from the desmosome back into the cytoplasm.

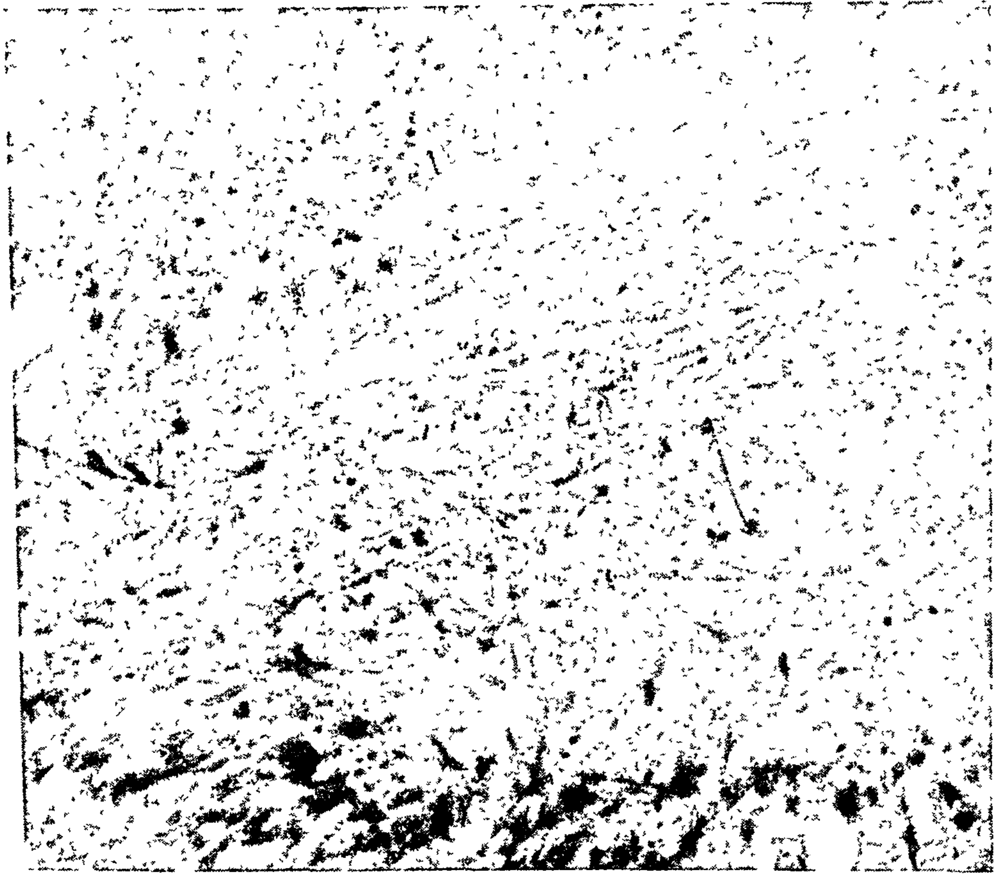


FIGURE 2 Portion of an epithelial cell from the upper stratum spinosum Tonofibrils are profusely distributed throughout the cytoplasm Small granules of approximately 90mμ diameter are dispersed among the fibrils ×9000

A comparison of the gingiva and cheek mucosa revealed that the demosomes were smaller and more delicate in the softer portions of the mucosa. In a typical field of the stratum spinosum from cheek epithelium, as seen in FIGURE 5, the tonofibrils did not appear to run as far back into the cytoplasm as in gingiva, though other cytoplasmic components, the mitochondria and electron dense granules, had a similar appearance. In addition to the minute contact plaques described above, the superficial oral epithelial cells were characterized by an extremely large cell surface, owing to the numerous foldings, elevations, and grooves, as reported in our previous electron microscopic observations mentioned above.

If a cell having many fingerlike projections extending from the outer cell wall were to be cut in a plane parallel to its surface and at the level of these projections, one would expect to see a series of disks. Such a field was noted frequently in sections of gingival epithelium, as illustrated in FIGURE 6. In passing it should be noted that in 1889 Kolliker suggested the possibility that ridges rather than fingerlike projections might be found on the surface of some oral epithelial cells. This possibility also has been discussed in recent electron

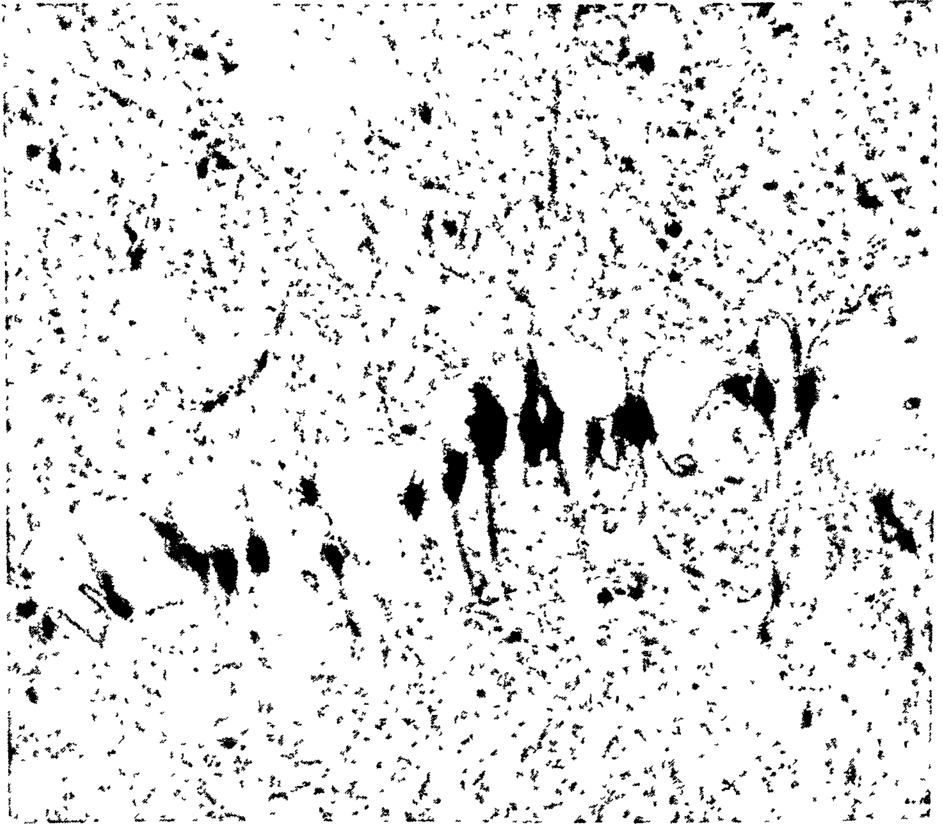


FIGURE 3 Border of 2 epithelial cells in the stratum spinosum of the human gingiva Tonofibrils can be seen extending from the desmosomes back into the cytoplasm The desmosomes appear quite dense compared to the other cytoplasmic structures ×9000

microscope literature by Fawcett (1958). In our own sections of oral tissues we have not seen any conclusive evidence of these interdigitating ridges, but must defer definitive interpretation until this subject is investigated further.

The keratohyalin granules are distinct entities of cornifying epithelium, yet the role of the granules in the process of keratinization remains a mystery. They may, indeed, have no connection with keratinization, although they do occur at the proper time and place in close association with the formation of keratin. The fact that they apparently contain no sulfhydryl groups, disulfide bonds, or other substances known to constitute sequential ingredients in keratin formation has been the main reason for their rejection as a precursor material. In heavily keratinizing oral epithelium, as in the mouse and rat, the dimen-

sions of the granules may be large and readily seen by optical microscopy. In the less keratinized oral mucosa of man these granules may be too small to be visualized except by electron microscopy (Sognnaes and Albright, 1956).

Typical keratohyalin granules in the granular layer of the human cheek mucosa were quite electron-dense and were found adjacent to cytoplasmic complexes consisting of vacuoles, small granules, and mitochondrial remnants, as illustrated in FIGURE 7. Ultrathin sections of the granular layer of normal

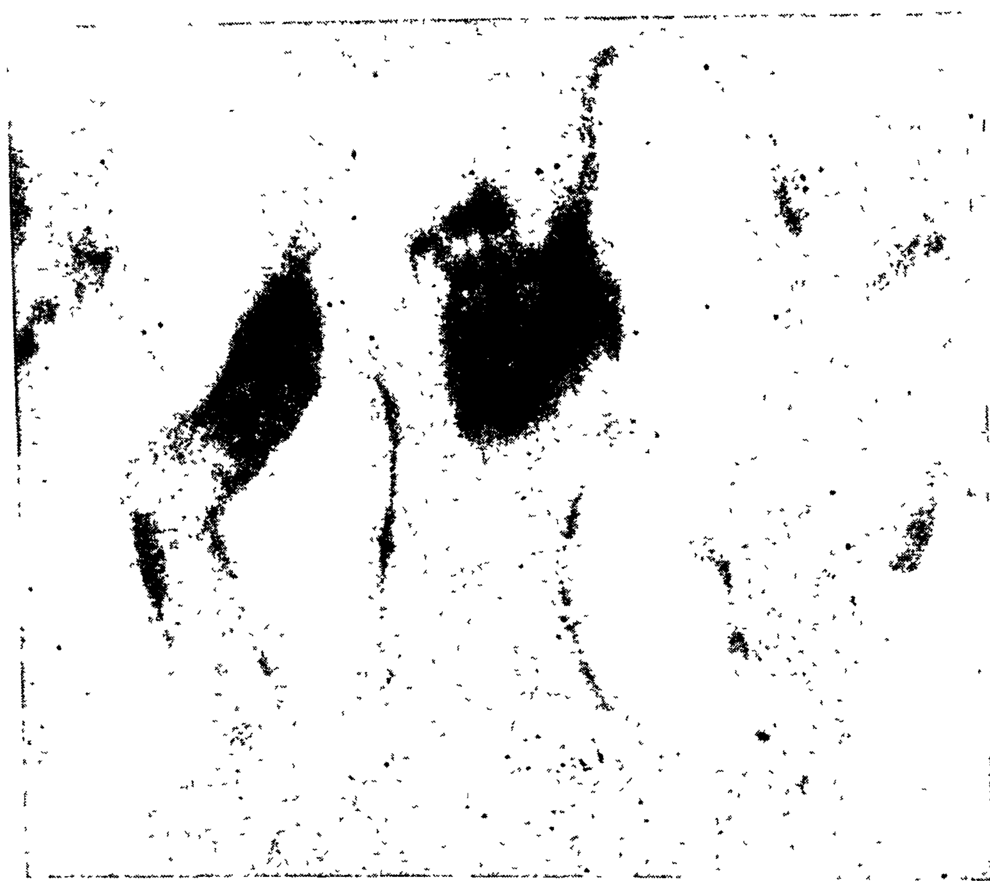


FIGURE 4 An enlarged view of an intercellular bridge from the stratum spinosum of human gingiva Note the highly organized desmosomes and the space between them. ×40,000

human gingiva revealed a honeycomb structure in the granules (FIGURE 8*a*) in contrast to the more homogeneous appearance of granules in the cheek mucosa. Higher magnification of the granules (FIGURE 8*b*) suggested that there are either spaces or areas of much lower density throughout the granules.

In the human gingiva and oral mucosa the outermost layer of cells are not keratinized as heavily as in lower animals such as the mouse In general, the oral epithelium, in contrast to skin, is subject to desquamation of whole cells, although in some instances the outside layer consists of little more than tough, electron-dense cell membranes. An example of such a heavily cornified area in the outer layer of mucosa, a section from the cheek of a two-month-old mouse, is illustrated in FIGURE 9. Indeed, this cornified oral epithelium more nearly

approaches that seen in skin. The cell remnants are pulled apart, the villi are more tortuous than is usual in human preparations, and the most electron-dense layers appear more granular than fibrillar.

Discussion

Currently there is considerable interest concerning the role of tonofibrils in keratinization. X-ray diffraction studies support an alpha-keratin diffraction pattern of these fibrils (Rudall, 1952). In an electron microscopic study of the

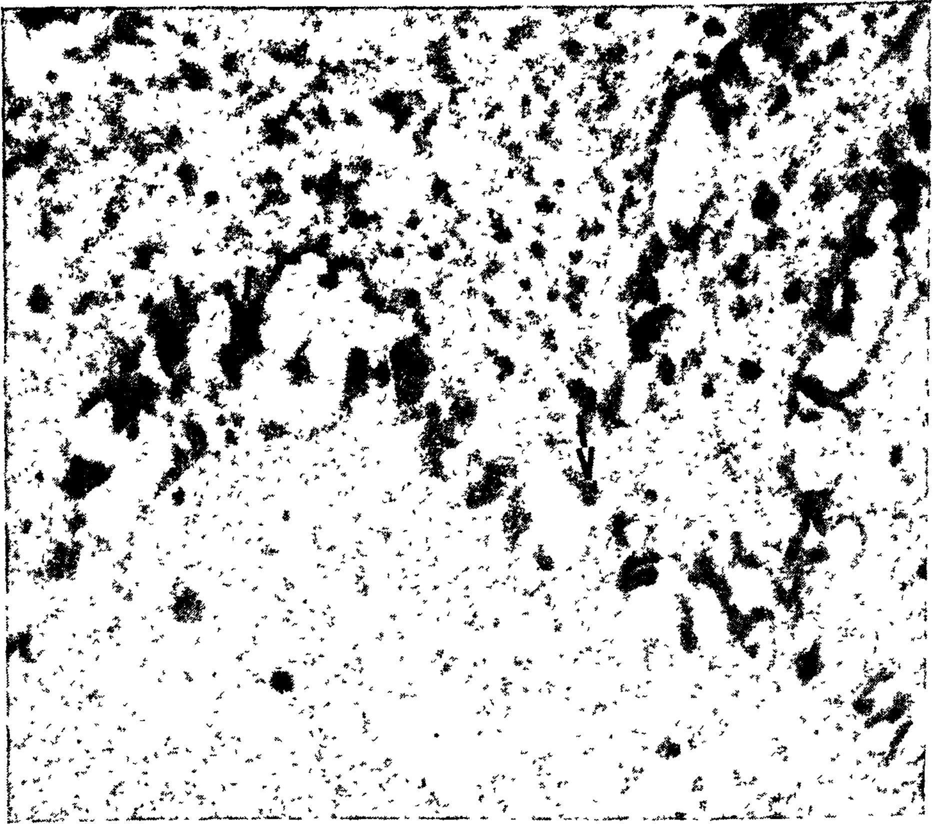


FIGURE 5 Cell borders from the stratum spinosum of human cheek mucosa The arrows mark typical desmosomes similar to those observed in gingiva Tonofibrils are not as prominent as those observed in gingiva ×8000.

human hair follicle, Birbeck and Mercer (1957) describe the initial appearance of fibrous keratin in the form of fine filaments in the cells of the mid and upper bulb. These filaments are similar in appearance and size to those seen in skin. It is unlikely, according to Montagna (1956), that tonofibrils are precipitation artifacts. Tonofibrils can be demonstrated by phase contrast microscopy (von Albertini, 1945), and the presence of SH— groups has been demonstrated.

Several other recent electron microscope studies on skin have mentioned the probable role of tonofibrils in the formation of skin keratin (Menefee, 1957; Brody, 1959; Charles, 1959). According to Selby (1955), in a study of skin, the tonofibrils are made up of bundles of tonofilaments having diameters of approximately 100 Å.

FIGURE 6 Gingival cells from the upper stratum spinosum. This section was cut tangentially to the surface villi, which therefore appear in the form of disks ×5000

FIGURE. 7. Normal human oral mucosa. Arrows mark typical keratohyalin granules which appear to be homogeneous in this preparation. ×22,500.

approaches that seen in skin. The cell remnants are pulled apart, the villi are more tortuous than is usual in human preparations, and the most electron-dense layers appear more granular than fibrillar.

Discussion

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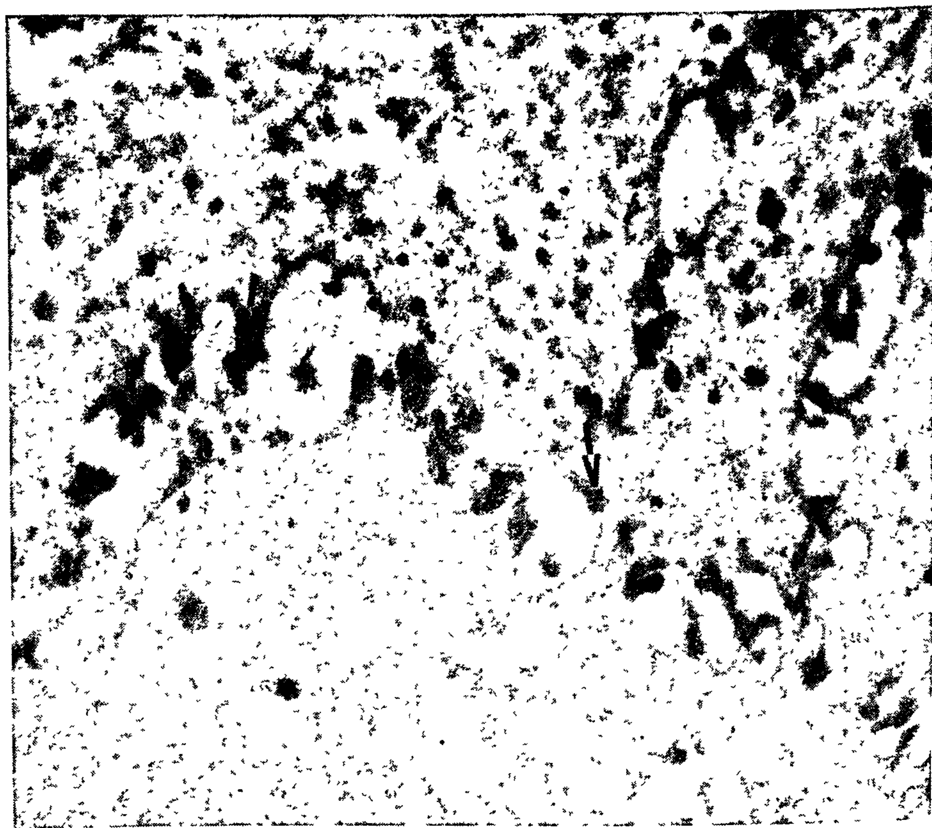


FIGURE 5 Cell borders from the stratum spinosum of human cheek mucosa The arrows mark typical desmosomes similar to those observed in gingiva Tonofibrils are not as prominent as those observed in gingiva ×8000

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One of the debatable morphologic features of epithelial cells in the skin and mouth are the so-called intercellular bridges. The lack of continuity of filaments or cytoplasm across the bridges in animal epidermis was noted first in a brief electron microscope report by Porter (1954). In fact, end-to-end contact was suspected by Bizzozero as early as 1870.

From an ultrastructural point of view, the structure of the attachment plaques or desmosomes, which make up part of the bridges, was described in detail with regard to skin by Odland (1958), who reported a total of as many as seven differ-

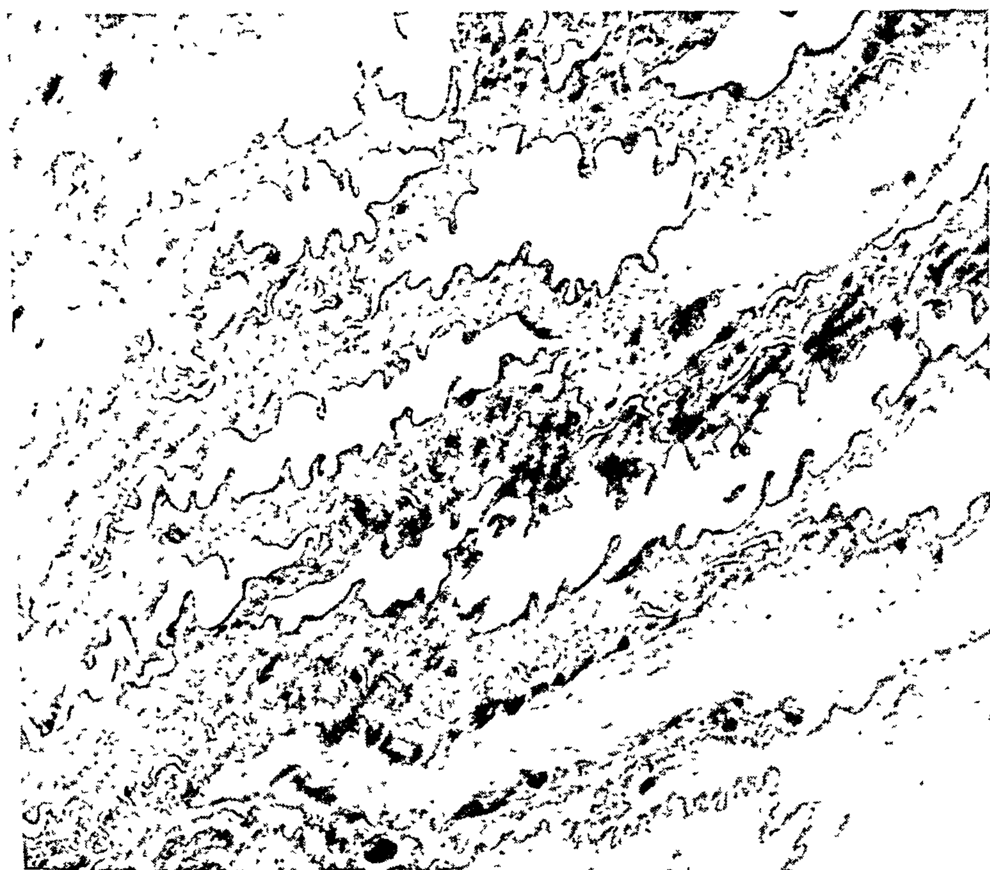


FIGURE 9 Cornified outer layer of epithelial cells from cheek mucosa of 2-month-old mouse Note tortuous interdigitations of cell membranes and dense keratinized cytoplasm ×5000.

ent layers of unidentified substance in the 300-Å space between the plaques. Hibbs and Clark (1959) referred to similar subdivisions when he noted that the desmosomes consisted of alternating dark and light laminae. Our own preparations from oral mucosa show attachment plaques very similar to those in Odland's publication of skin, but our findings are not sufficiently detailed to justify comment about the laminae between the plaques.

The significance of the fibrillar network or honeycomb structure that we observed in the keratohyalin granules from gingiva is unknown. As mentioned above, the granules are often seen adjacent to degenerating mitochondria. Such altered mitochondria have been proposed as being involved in keratohyalin formation by Sheldon and Zetterquist (1956). I have noted fragmentation of

In all of our own specimens of human gingiva an abundance of tonofibrils was observed. It is not yet clear how these vary in different mouth epithelia of the same species or how they may vary in different species. For one thing, the tonofibrils were not as prominent in our sections of cheek oral mucosa from

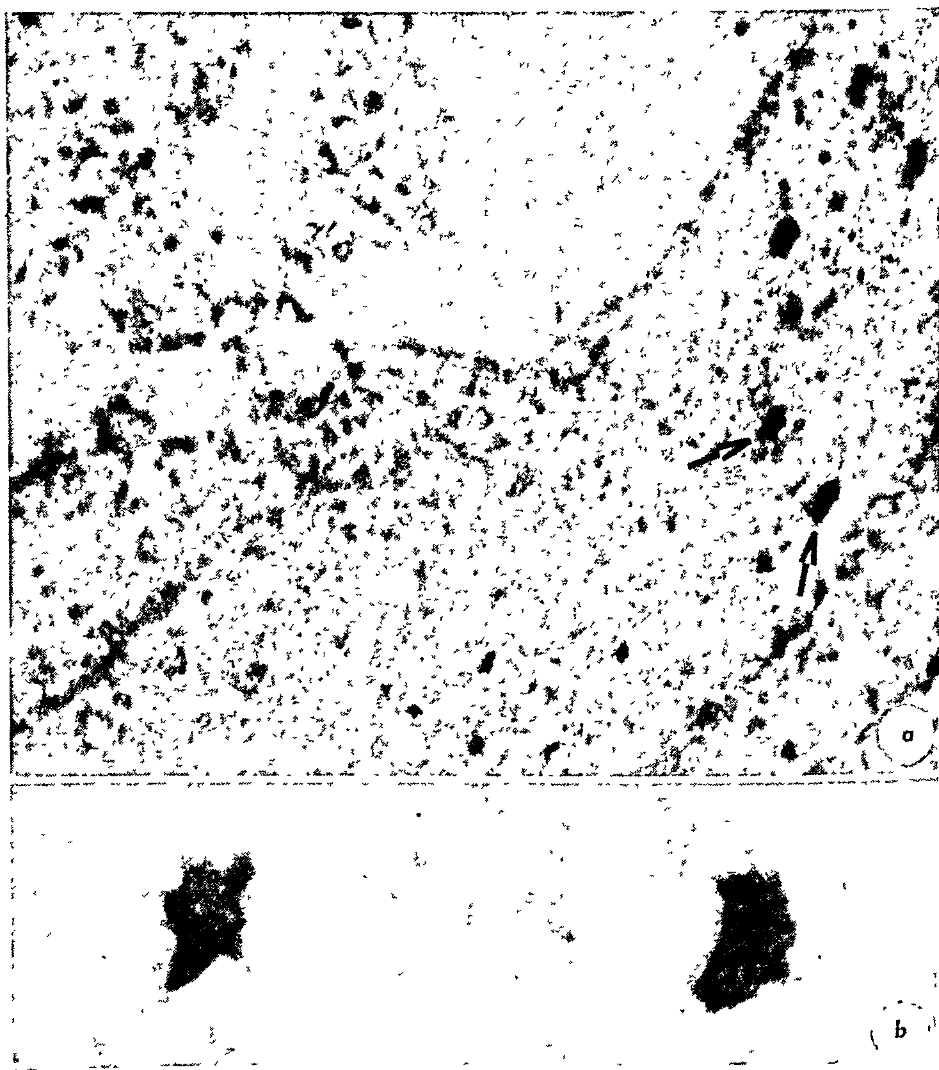


FIGURE 8 (*a*) Cells from the granular layer of normal human gingiva Numerous electron-dense keratohyalin granules are present, which show suggestions of internal structure ×8000 (*b*) High magnification of keratohyalin granules from a field similar to that of *a* The granules are seen to have a honeycomb internal structure ×32,000

humans as from mice. Conversely, Montagna (1956) mentions the fact that tonofibrils were scanty in the skin of the mouse

Menefee (1957), discussing keratinization in the epidermis of embryonic mice, noted tonofilaments about 40 Å in diameter with alternating densities along their length suggesting granularity. Brody (1959), on the other hand, pictured keratin in the corneum of guinea pig skin as either tubelike or consisting of (unstained) filaments in a darker matrix

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the nucleoli and deep foldings of the nuclear membrane in conjunction with the appearance of keratohyalın granules along the nuclear surface, but these and other assocıations may be coincidental. Further studies must be made to determine the nature of the keratohyalin granules and their relation to the keratinization process.

Summary

Thin sections of epithelium from normal human gingiva and cheek mucosa were examined by electron microscopy.

Tonofibrils, consistıng of tonofilaments with a diameter of less than 200 Å, were observed in both cheek mucosa and gingiva, the tonofibrıls of the gingıva being particularly abundant.

No contınuity of any cytoplasmic structures existed across the so-called intercellular bridges. The union of two cells at the bridge area consisted of a paır of electron-dense attachment plaques or desmosomes separated by a distance of approxımately 300 Å.

Ultrathin sections of keratohyalin granules in the gingiva frequently revealed a honeycomb internal structure ın contrast to previously observed granules whıch had appeared to be homogeneous

The possible relations of these epidermal structures to the process of keratınization were discussed.

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involvement of the circumvallate papillae was observed. The process begins with impairment of keratinization, followed by parakeratosis, hydropic degeneration, and occasional rarefaction of the underlying submucosa. Later, the papillae disappear completely, and the dorsum becomes covered by a thin layer of smooth atrophic epithelium.

The pathological findings in successive attacks of the deficiency may not be identical This is demonstrated especially well in riboflavin deficiency. While biopsies taken early during the first attack failed to reveal any specific pathological findings, biopsies taken during the second attack showed progressive



FIGURE 1 Niacin deficiency Dorsum of the tongue Filiform papillae are seen undergoing various stages of atrophy. The fungiform papillae disappear later. Some of the areas are completely devoid of papillae and are covered by a thin layer of smooth atrophic epithelium

changes in epithelial and connective tissue. The same is true of the nerves, which may appear normal during the first attack and exhibit signs of degeneration during the second.

These features are common to the deficiencies studied: the degree of lingual involvement parallels the severity of the deficiency; the extent of the lesions and the degree of degeneration depend upon the duration of the deficiency and upon the number of successive attacks of acute deficiency experienced; the response to specific vitamin therapy is prompt; and, irrespective of the type of deficiency, the most striking microscopic changes are found in the epithelium. Definite early changes in the connective tissue were observed only in riboflavin deficiency.

The development of glossitis is preceded by nonspecific symptoms that include loss of weight, irregularities in appetite, and gastrointestinal disturbances.

EXPERIMENTAL ANIMAL STUDIES OF TONGUE CHANGES IN NUTRITIONAL DISEASE*

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Introduction

The metabolic disturbances, especially those caused by nutritional deficiencies, frequently leave their imprint in the oral cavity. Among the nutritional factors, the following have been found important for maintenance of healthy oral mucosa. niacin, riboflavin, pyridoxine, pantothenic acid, folic acid, vitamin B_{12}, thiamin, vitamin A, vitamin C, iron, and extrinsic factor. It is of interest to note that, with the exception of vitamin C, all of the above factors affect the tongue; it is here that the most characteristic oral lesions are found.[1,2]

My observations on malnourished human beings[3,4] stimulated my interest in this problem, and the study was extended to include production of experimental deficiency states in animals [5-7]

Experimental Procedure

The deficiencies in niacin, riboflavin, pyridoxine, pantothenic acid, and folic acid were studied in young dogs placed on a synthetic diet.[5,6] The animals were kept on their respective rations until definite signs of the deficiency became manifest On reaching this stage they were given, parenterally or orally, the withheld vitamin, and the response was observed During the experimental period lasting from 140 to 500 days, this procedure was repeated up to five times in the same dog. This gave a good opportunity for the study of initiation of the lesions, their progression, and response to specific therapy.

The observations included examination of the oral cavity, biopsy, and general observations such as weight and gastrointestinal activity. Since anemia and changed gastric acidity also may play a role in the etiology of glossitis, blood examination and gastric analysis also were included.

Results

Oral Lesions

Lingual lesions. The most common lesions observed were found on the dorsum of the tongue They were noninflammatory and were characterized by papillary atrophy and degeneration, predominantly involving the epithelium

Grossly, this type of glossitis accompanying the deficiency states studied cannot be differentiated This is especially true of the patchy lesions seen in riboflavin and pyridoxine deficiencies Occasionally the lesions may be so inconspicuous as to preclude their detection on casual examination. While in most cases the involvement was restricted to the anterior two thirds of the tongue, in niacin deficiency the whole dorsum may be involved The filiform papillae are usually the first to be affected, followed by the fungiform papillae. No

* The work reported in this paper was supported in part by a grant from the National Institutes of Health, Public Health Service, Bethesda, Md



FIGURE 5. Biopsy obtained during the first attack of riboflavin deficiency. The area is devoid of filiform papillae. Single fungiform papillae show atrophy. Epithelium is almost normal.

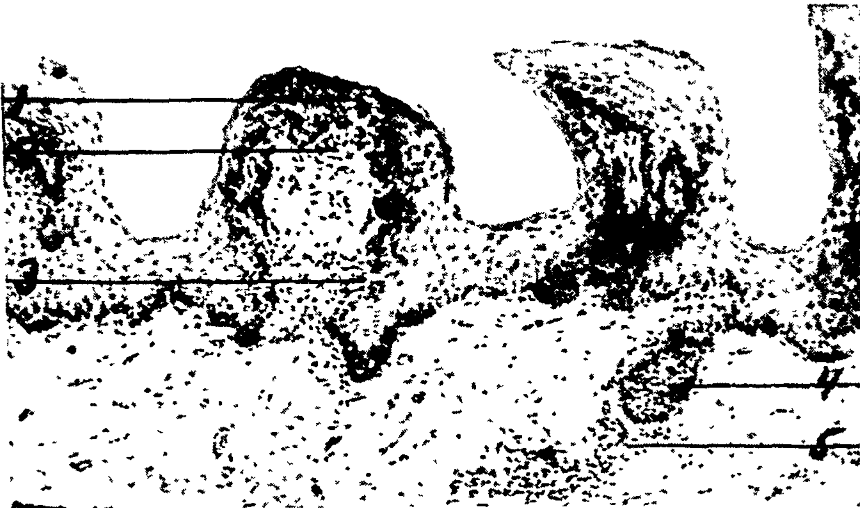


FIGURE 6 Second attack of riboflavin deficiency There is degeneration of epithelium and taste buds[1] The basement membrane is destroyed.[3] Foci of cellular infiltration may be present.[5]



FIGURE 7. Second attack of riboflavin deficiency. Picture shows advanced stage of epithelial degeneration There are numerous and extensive breaks in the basal layer and complete disintegration of the deeper part of epithelium.



FIGURE 2 Normal dorsum of the tongue A fungiform papilla with two taste buds is seen surrounded by filiforms that are regular in shape and size. They are covered by well-keratinized epithelium



FIGURE 3 Niacin deficiency. Atrophy of papillae These changes are not pathognomonic of this deficiency state



FIGURE 4 Pyridoxine deficiency Late stage of papillary atrophy. The epithelium is smooth and atrophic. A similar picture is seen in other deficiency states



FIGURE 5. Biopsy obtained during the first attack of riboflavin deficiency. The area is devoid of filiform papillae. Single fungiform papillae show atrophy Epithelium is almost normal.



FIGURE 6. Second attack of riboflavin deficiency There is degeneration of epithelium and taste buds[1] The basement membrane is destroyed.[3] Foci of cellular infiltration may be present.[5]



FIGURE 7 Second attack of riboflavin deficiency. Picture shows advanced stage of epithelial degeneration. There are numerous and extensive breaks in the basal layer and complete disintegration of the deeper part of epithelium.

Anemia also may appear early in the course of the deficiency, and for a considerable time it may not be accompanied by evident lingual lesions. However, no definite relationship could be established between the degree or duration of anemia and atrophic changes in the lingual epithelium.

On therapy, the gastrointestinal symptoms are the first to disappear. The regeneration of the lingual papillae could be observed grossly within 2 to 3 days, and complete regeneration of the papillary layer within one week. The blood changes were the last to disappear.

Since it has been found that the degenerative changes are more pronounced with each successive attack of deficiency, it may be expected that a deficiency of lesser degree, although acting over a longer period of time, will result in a more extensive damage, and that a multiple vitamin B deficiency will be accompanied by more severe lesions than those accompanying a deficiency in any one B vitamin. The lesions peculiar to various deficiencies become superimposed It becomes understandable that the glossitis observed in vitamin B deficiency in human beings may fail to respond, or responds incompletely to the administration of a single B vitamin.

Gingival lesions With the exception of one niacin- and one pyridoxine-deficient dog, tooth brushing was found to be effective in preventing the development of gingivitis. In nonbrushed areas the degree of gingivitis roughly paralleled the amount of calculus and food debris present. Grossly and histologically, the gingival lesions observed in the animals consuming various deficient diets were nonspecific and could not be differentiated one from the other nor from the lesions observed in the control animals. The difference was in the degree of the involvement, which did not bear a clear relation to the type of deficiency

In none of the animals was pocket formation observed grossly, roentgenographically, or histologically. There was some atrophy (thinning) of the alveolar bone that was especially marked in one of the niacin-deficient dogs. This deficiency also was accompanied by bleeding from the marginal gum, redness and sensitivity of the oral mucosa, and small ulcers on buccal mucosa and borders of the tongue

Other Findings

Blood picture. The anemia present in the deficient animals was hypochromic and either normocytic or microcytic in type. The most severe anemia was observed in niacin deficiency in which poikilocytosis and anisocytosis of the red cells in the blood stream and bone marrow were prominent features. The bone marrow was hyperplastic in niacin deficiency and hypoplastic in other deficiencies studied There were no appreciable changes in the white cell counts

Blood volume The blood volume in dogs was found to constitute from 8.0 to 9.0 per cent of the body weight, being distributed about equally between the plasma and cells. The variations in the blood volume were due predominantly to a decrease in the cell volume (anemia) until dehydration supervened during the late stage of the deficiency. The fluctuations in the plasma volume tended to parallel the severity of the deficiency state.

Gastric acidity. The acidity of the histamine-stimulated gastric juice remained normal in all deficient animals.

Conclusions

The most common oral manifestation of niacin, riboflavin, pyridoxine, pantothenic acid, and folic acid deficiencies in the dog is a glossitis characterized by noninflammatory and nonspecific atrophy of the dorsal mucosa of the tongue. No relation could be found between the development of glossitis and the blood picture or gastric acidity accompanying these deficiency states. Basically, the lesions are similar to those observed in deficient human beings, and the same B vitamins have been found to be effective in their cure.

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CHANGES OF THE HUMAN TONGUE IN PROTEIN AND VITAMIN DEPLETION AND REPLETION*

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Nutritional deficiencies observed in man are usually due to a deficient intake of several vitamins as well as of proteins. The fully developed clinical manifestations of the deficiency signs are probably the result of long-standing inadequate intake of essential components of the diet Relatively little is known about the speed of the development and the sequence of the appearance of the various stages which result in the clinically recognizable, typical deficiency changes. Little information is available on the time interval needed for the reversal of the abnormal changes following administration of vitamins and proteins. The sequence of events in the stages of dietary depletion and repletion was studied under controlled conditions in patients receiving a protein- and vitamin-deficient diet for therapeutic purposes on the Metabolic Research Unit of the Division of Neoplastic Diseases of Montefiore Hospital.

Material and Methods

Of the patients under study on the Metabolic Research Ward, three were selected who were in good physical condition at the start of the study and whose physical status was similar at the end of the study following the institution of vitamin and protein repletion The patients were observed under strictly controlled dietary conditions

Patient 1 was a 53-year-old female on whom a diagnosis of chronic myelogenous leukemia was made 1 year prior to this study. At the time of the study, the patient was in good nutritional state but pale. The body weight was 55.4 kg. The principal abnormal clinical findings were enlargement of the liver and spleen and slight enlargement of the axillary and inguinal lymph nodes Laboratory findings: Hgb 5.7 gm., RBC 2.2 million, WBC 540,000, polymorph nuclear cells 8 per cent, early granulocytic forms 19 per cent, myelocytes 38 per cent, metamyelocytes 6 per cent, blast cells 8 per cent, lymphocytes 12 per cent Bone marrow aspiration showed marked myeloid hyperplasia, the cells of the myeloid to erythroid series having a ratio of 25.1. Blood chemistries, electrocardiograms, and roentgenograms were normal.

Patient 2 was a 42-year-old male on whom the diagnosis of a reticulum cell sarcoma of the left upper lobe of the lung was made by thoracotomy and histological tissue examination 6 months prior to this study The patient was ambulatory and in good physical condition, body weight 69.8 kg. Except for a slight shift of the trachea to the right and the presence of several small lymph nodes in the left supraclavicular area, no physical abnormalities were noted. The blood count, the blood sugar, blood urea nitrogen, bone marrow aspiration, and the electrocardiogram and roentgenograms, other than of the chest, were normal.

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Patient 3 was a 62-year-old female on whom a diagnosis of lymphosarcoma was made on lymph node biopsy. At the time of the study, the patient was in good physical condition, and the only physical abnormalities were enlargement of the axillary, inguinal, and epitrochlear lymph nodes. The body weight was

TABLE 1
FOODS OF THE DEFICIENCY DIET

Polished rice, 300 gm dry weight per day
Fruit juice (grapefruit, pineapple, grape, apple)
Jelly
The fruits and juices of this diet were selected because of low vitamin B content.
Each 100 gm. rice was cooked in 400 ml water for 20 min at a maintenance temperature of 92° C. Rice was offered with the water in which it was cooked
All food elements were taken from the same source

TABLE 2
COMPOSITION OF DEFICIENCY DIET

Calories	2550
Protein	26 gm
Carbohydrate	608 gm
Fat	1 7 gm
Calcium	151 mg.
Phosphorus	432 mg
Sodium	7 1 mEq
Potassium	50 1 mEq

TABLE 3
VITAMIN CONTENT OF THE DEFICIENCY DIET
(Calculated)

Vitamin	Content	Fraction of daily minimal requirements
A	2294 Int. U	1/2
B_1	408 μg	1/3
B_2	274 μg.	1/10
Niacin	6 mg	1/2
Pantothenic acid	1733 μg	?
Pyridoxine	0	0
Biotin	0	0
Ascorbic acid	69 mg.	3/4
D	0	0
Folic acid	?	?

73.8 kg ; the blood count, bone marrow aspirations, urinalysis, blood sugar, blood urea nitrogen, and roentgenograms of the chest and of the skeleton were normal.

The diet consisted chiefly of rice and fruit, except for fruits having a high content of B vitamins (TABLE 1) The dietary intake was adequate in calories, but deficient in proteins and in vitamins No vitamins or iron were supplemented to the diet. The composition of the daily dietary offerings is listed in TABLE 2. The vitamin content of the diet and the approximate fractions of the minimal daily vitamin requirements are listed in TABLE 3.

The fluid intake and output, the body weight, the urinary creatinine, calcium, and phosphorus were determined daily, and the metabolic balances of calcium, phosphorus, nitrogen, sodium, and potassium were measured during the entire study. The duration of the protein- and vitamin-deficient dietary intake was 126, 105, and 98 days in the 3 patients, respectively. Although the amount of the diet as outlined above was offered to the patients, the dietary intake decreased during the course of the study due to rejection of food secondary to anorexia the longer the deficiency diet was continued. In order to hasten the onset of the dietary deficiency, Pteropterin, 35 mg., was given twice a day intravenously, after 36 and 24 days of the deficiency diet, respectively, and was continued for 60 days and 36 days in Patients 1 and 2. Patient 3 did not receive

TABLE 4

DURATION OF INTAKE OF DEFICIENCY DIET AND TYPES OF VITAMINS ADDED

Patient	Age, sex	Diagnosis	Deficiency diet (days)		Vitamins added
			Before*	After†	
1	53 F	Chronic myelogenous leukemia	126	26	Plebex (Winthrop), 2 cc; Folic acid, 1 cc } I M /day B_{12}, 25 μg I M twice per week
2	43 M	Reticulum cell sarcoma of lung	105	22	Plebex (Winthrop), 2 cc; Pyridoxine, 100 mg; Vitamin C, 300 mg } on 3 successive days, I V, in 1000 ml 5% glucose in water Followed by Hexavitamin, 3 tablets per day, and crude liver extract, 1 cc., every other day.
3	62 F	Lymphosarcoma	98	33	Plebex (Winthrop), 2 cc; Folic acid, 1 cc } I M /day B_{12}, 25 μg. I M twice per week

* Before vitamin administration
† After the start of addition of vitamins

this antimetabolite. Radiotherapy was given to all 3 patients Patient 1 received a total of 100 r total body radiation over a 26-day period starting on the ninety-sixth day of the deficiency diet Patient 2 received local irradiation to the left upper anterior and posterior chest with cobalt-60 teletherapy unit for a 24-day period after he had been receiving the deficiency diet for 95 days (total tumor dose 42.2 r). Patient 3 received local radiotherapy to several areas of lymph node enlargement for 56 days starting on the twenty-fourth day of the intake of deficient diet. This diet was continued in all 3 patients during the courses of radiotherapy. When the effect of the deficiency diet was most marked, repletion was instituted by adding vitamins to the dietary intake without changing the constituents of the deficiency diet The route of administration and the amounts of vitamins given depended on the severity of the deficiency state. The added vitamins are listed in TABLE 4, the composition of Plebex in TABLE 5, that of Hexavitamins in TABLE 6.

Photographs of the tongue were taken prior to the institution of the deficiency

diet, at weekly intervals during the administration of this diet, and in short succession in the stage of repletion. Biopsies of the tongue were taken for histological examination whenever possible. The correlation between the gross appearance and histopathology of the tongue previously reported[1] served as a basis for the interpretation of the finding.

Metabolic balances of calcium, phosphorus, nitrogen, sodium, and potassium were performed on aliquots of 6-day pools of the excreta (urine and stool) and on aliquots of the diet.

TABLE 5
COMPOSITION OF PLEBEX
(Per ml)

Thiamine hydrochloride	10 mg
Riboflavin	2 mg
Niacinamide	100 mg
Pyridoxine hydrochloride	5 mg
Calcium pantothenate	5 mg

TABLE 6
VITAMIN CONTENT OF HEXAVITAMIN
(Per tablet)

Vitamin A	5000 U S.P units
D	400 U S P units
C	75 mg
B_1	2 mg.
B_2	3 mg
Nicotinamide	20 mg

Results

PATIENT 1

The mucosa of the cheeks was pale and slightly edematous; minimal marginal gingivitis was present; the filiform papillae were normal in length and distribution except for the tip of the tongue, where they were shorter, therefore, the fungiform papillae were more visible than usual The fungiform papillae, however, were of normal color

FIGURE 1 shows the gross picture of the tongue of Patient 1 prior to institution of the restricted diet.

Period of Depletion

Objective findings. No significant changes occurred for 38 days of the regimen of strict deficient dietary intake of the proteins and vitamins. At that time, the first changes involving the tip of the tongue were noted there was shortening of the filiform papillae, and the fungiform papillae became more visible, but were not prominent. Irregular transverse and longitudinal fissures appeared in the center of the tongue 10 days later, and there was further reduction in number of the filiform papillae (FIGURE 2). These changes became more

prominent by the seventy-first day (FIGURE 3). By the seventy-seventh day of the deficient diet, the changes had progressed further, with more noticeable shortening of the filiform papillae, shedding of the superficial layers of the epithelium in the center of the tongue, and covering of some areas by a grayish-yellowish coating. The mucosal surface of the tip of the tongue and the lateral



FIGURE 1 Patient 1, before deficiency diet

margins became smooth due to desquamation of the epithelium. The previously visible fungiform papillae became less visible and flatter, and cheilosis was noted for the first time. The deficiency signs progressed very rapidly from the seventy-seventh day of deficient intake· 4 days later, the deficiency signs were even more marked, the tongue became beefy red, and the filiform papillae had disappeared (FIGURE 4). These severe signs of dietary deficiency progressed even further within the next 45 days, during which the patient was observed at 9 different times. The tongue remained beefy red, the filiform papillae were

FIGURE 3. Patient 1, 69 days of deficiency diet.

FIGURE 2. Patient 1, 45 days of deficiency diet.

completely absent, and the fungiform papillae appeared slightly enlarged and cystic in the center of the tongue, giving the appearance of small blisters (ninety-first day). An indication of cobblestone appearance of the mucosa of the tongue was first present on the one hundred and twenty-second day and continued to be present for the next twenty-one days. In addition to the findings described, a few small islands grayish in color and suggestive of small areas of keratinization were noted in the center of the tongue on the one hundred and twenty-sixth day of dietary deficiency (FIGURE 5). The tongue also became very pale, probably due to anemia



FIGURE 4 Patient 1, 91 days of deficiency diet.

Subjective changes. The patient started to complain of discomfort in swallowing and taste disturbances by the seventy-seventh day of restricted diet, and of burning of the tongue and lips and dryness of the mouth by the eightieth day. She noted a fishy taste even on ingesting sweet nutrients and imbibing fruit juices by the eighty-sixth day, and further difficulties in swallowing and burning

along the area of the esophagus were reported by the ninety-first day of deficiency diet. Marked decrease of taste appreciation occurred, and a feeling of marked dryness of the tongue was present from the one hundred and third day on and became most severe after 124 days of dietary restriction.

Period of Repletion

Objective findings. After 129 days of dietary deficiency, vitamins were added to the deficiency diet for 26 days (TABLE 4). Four days after the institution



FIGURE 5 Patient 1, 128 days of deficiency diet.

of vitamin therapy, there was a decrease of the severity of the cheilosis. The tongue became slightly more pinkish and wet, but there was no regrowth of the filiform papillae. The fungiform papillae were visible but not prominent (FIGURE 6). One day later, the findings were essentially the same. Nine days after the addition of vitamins, filiform papillae were noted but appeared very short, the fungiform papillae appearing the same as on days 4 and 5. The same findings were noted on the twelfth day of vitamin supplementation. The scar of the tongue biopsy, taken 150 days earlier, became visible. After 15 days of vitamin addition, the filiform papillae had developed over almost the entire surface of the tongue except for the area of the tip, in addition, the cheilosis had



FIGURE 6 Patient 1, 4 days after addition of vitamin to deficiency diet

FIGURE 7 Patient 1, 22 days after addition of vitamin to deficiency diet

further decreased. The return to a normal mucosal pattern continued in the next few days, and the tongue became normal on the twenty-second day of vitamin supplementation (FIGURE 7). The filiform papillae were moderately long, especially in the center, but were shorter at the tip; the surface of the tongue became reddish-silvery grey, indicative of recornification of the epithelium; and the only remaining abnormality was slight reddening of the tip. The cheilosis had considerably decreased and was in the stage of healing.

Subjective changes. Disturbances of taste had improved slightly after 4 days of vitamin supplementation, improved further between the eighth and twelfth

TABLE 7
DESCRIPTION OF SEQUENCE OF CHANGES OF TONGUE OF PATIENT 1

Time in days from start of deficiency diet	Observations
38	Beginning changes on tip of tongue
	Slight fissures
45	Increasing changes on tip of tongue
	Increased fissures
66	No change, no cheilosis
77	Indication of cheilosis, tongue getting bald
	Taste disturbance (fishy), difficulty in swallowing
80	Increased cheilosis, increased baldness of tongue
	Burning of tongue, lips
91	Burning in esophagus
	Difficulties in swallowing
122	Height of changes
	Slight cobblestone appearance, fusion
126	Tongue wet, feels dry to patient
	Slight cobblestone appearance
Time in days from start of vitamins	**Vitamin therapy started**
4	Tongue light pinkish
	Perlèche less, no gingivitis
12	Taste sensation improving
	Tongue light pinkish
15	Filiform papillae developing
	Perlèche slight, not yet quite normal taste
22	General improvement

days of vitamin therapy, and had subsided completely by the fifteenth day. The sensation of burning of the tongue and discomfort on swallowing had improved gradually during the treatment period and had disappeared completely by the twenty-second day of vitamin intake.

The sequence of the changes of the tongue of Patient 1 is listed in TABLE 7.

PATIENT 2

Period of Depletion

Objective findings. Approximately 1 month following the institution of the deficiency diet, beginning changes of the tongue were noted (FIGURE 8). The filiform papillae became shorter at the tip of the tongue, but remained un-

changed at the center. The fungiform papillae became visible at the anterior third of the tongue, and the margins became slightly smooth and had a dark red hue. Fourteen days later (44 days of deficiency diet) the changes on the anterior third of the tongue became more prominent. The filiform papillae became very short and blunt; the fungiform papillae were visible and became more red. On the fifty-third day of deficiency diet, the tongue was dry and



FIGURE 8. Patient 2, 30 days of deficiency diet

red, and the filiform papillae became short and blunt also in the center of the tongue, so that the area of shortening of papillae extended from the tip in the shape of an inverted U into the center of the surface of the tongue (FIGURE 9). The fungiform papillae were more prominent, and the development of cheilosis was noted The color of the tongue and of the buccal mucosa became dusky red and edematous. On the seventy-seventh day of the study, these changes had progressed further, so that only a narrow rim of normal mucosa was recognizable on the side of the tongue. Cheilosis was well developed at that time. Five examinations were performed in the succeeding 28 days to reveal the same

type of findings, the only additional changes being further diminution of the remaining narrow areas of normal mucosa in which the filiform papillae also became extremely short.

Subjective changes. The patient started to notice loss of appetite and burning of the tongue on the fifty-third day of the deficiency diet and loss of taste on the sixty-third day. The patient stated that the changes in taste sensations



FIGURE 9 Patient 2, 67 days of deficiency diet

were aggravated by the injections of Pteropterin and improved when this medication was omitted, so that the disturbances of taste appreciation had greatly diminished by the seventy-seventh day of the deficiency diet, at which time Pteropterin had been discontinued for 10 days. However, the appetite had remained extremely poor for the remaining period of the deficiency diet intake.

Period of Repletion

Procedure. The deficiency state was at its height on the one hundred and twelfth day of the rice and fruit diet. The patient felt extremely weak and was

unable to get out of bed, and the blood pressure was 80/40 mm. Hg. The patient was given very large doses of vitamins intravenously on 3 successive days (TABLE 4). Subsequently, Hexavitamins, tablets 3, were given 3 times per day orally. The deficiency diet was continued during the phase of vitamin administration. No other therapy was given.

Objective findings. The blood pressure increased to normal levels within several hours of the intravenous administration of vitamins; the patient felt much improved after 1 day of vitamin administration and was able to get out of bed. A few filiform papillae reappeared after 24 hours of treatment and, 4 days after the start of vitamin administration, remarkable changes were noted. The tongue was now wet, the cheilosis had disappeared, and the color of the oral mucosa had changed from red to dark pink. There was redevelopment of the filiform papillae on the entire surface of the tongue, although they were still short. The fungiform papillae were still large but not prominent, and their color was that of the tongue and not as bright red as was noted in the phase of deficiency diet. On the seventh day following the institution of vitamin administration, beginning cornification of the epithelium of the tongue was noted, and the mucosa appeared normal although the color was still slightly red. On the fourteenth day of treatment, the filiform papillae were of normal length except over the tip of the tongue, where they were still slightly shortened. Further return to normal was noted 4 days later. When the patient was re-examined on the thirty-fifth day of vitamin administration, the tongue was completely normal (FIGURE 10).

Subjective changes. The general condition improved rapidly, and the patient felt much stronger within one day of administration of large doses of vitamins by the intravenous route. The burning of the tongue and the difficulty in swallowing were greatly relieved four days after the institution of vitamin treatment and continued to improve further during this regimen.

The sequence of events noted in the depletion and repletion phases of Patient 2 is listed in TABLE 8.

PATIENT 3

Period of Depletion

Objective findings. Prior to the institution of the deficiency diet, the tongue was normal, of pink color, and well hydrated. However, the filiform papillae appeared somewhat shortened, especially on the tip of the tongue. The fungiform papillae were normal in color and size. After 15 days of intake of the deficiency diet, the tongue had a beefy red color, and the number of filiform papillae decreased on the tip of the tongue and became shorter in the center of the tongue. Minimal cheilosis became apparent. These findings remained approximately the same for the next 50 days, except that coating of the center had been noted since the thirty-fifth day and dryness of the tongue since the fiftieth day of the deficiency diet. A further progressive decrease of the number of filiform papillae in the center and margin of the tongue was noted between the fiftieth and sixty-third day of the deficiency diet. These findings continued to be observed during the entire period of dietary deficiency, except that the

tongue started to have a cobblestone appearance by the seventieth day of dietary restriction. The mucosa of the oral cavity decame dusky red and slightly edematous by the eightieth day of diet. The total duration of the deficiency diet intake without supplementation of vitamins was 98 days.



FIGURE 10 Patient 2, 35 days after addition of vitamins to deficiency diet.

Subjective changes. Since this patient did not volunteer any information regarding the subjective changes and mentioned only occasional disturbances of taste appreciation, no statement as to subjective changes in the phase of depletion or repletion can be made.

TABLE 8

DESCRIPTION OF SEQUENCE OF CHANGES OF TONGUE OF PATIENT 2

Time in days from start of deficiency diet	Observations
32	Beginning changes of tip of tongue
46	Loss of appetite
	Burning of tongue, more changes of tip
53	Taste disturbance; "loses" taste when getting antifolic, taste returns when injections stop (Pteropterin)
	Tongue dry, red, red part includes tip and center
	Indication of perlèche
	Filiform papillae short at tip and center, fungiform papillae slightly prominent
67	Tongue dry, red, area of fairly well-hornified filiform papillae greatly reduced
	Off antifolic, taste back
	Perlèche well developed
	Fungiform papillae at tip slightly enlarged, not reddened
95–105	No normal filiform papillae of tongue
	Some fissuring
	Fungiform papillae slightly prominent, not reddened
Time in days from start of vitamins	**Vitamin therapy started**
4	No perlèche; color of tongue changing toward normal
	Filiform papillae seem to redevelop all over tongue
	Swallowing easier
7	Increasing keratinization; tongue wet
14	Increasing keratinization
	Fungiform papillae small, not reddened
	Cheek almost normal, with slight indication of edema
21	Further increase of keratinization, condition of tongue similar to that at start of study
35	Patient discharged

TABLE 9

DESCRIPTION OF SEQUENCE OF CHANGES OF TONGUE OF PATIENT 3

Time in days from start of deficiency diet	Observations
0	Slight shortening of filiform papillae, tip of tongue
14	Slight indication of perlèche
	Slight shortening of filiform papillae in center, starting to decrease on tip
	Beefy red color of tongue
35	Coating of tongue, slight reddening of corners of mouth
49	Dryness of tongue, slight cheilosis
68	Filiform papillae almost disappeared
	Tongue beefy red
77	Cobblestone tongue, cheilosis
84–97	Same as above, but tongue became much smoother
Time in days from start of vitamins	**Vitamin therapy started**
4	Tongue wet, less red
10	Beginning growth of filiform papillae, center of tongue
17	Filiform papillae reappeared on tip of tongue
20	Color of tongue normal
33	Tongue normal except for persistent slight shortening of filiform papillae on tip and margins

Period of Repletion

Objective findings. The first observation was made 2 hours following institution of vitamins. The findings were the same as prior to vitamin administration except that the color was somewhat lighter. An indication of the re-

TABLE 10
APPEARANCES OF DEFICIENCY CHANGES OF THE TONGUE

	Days from start of deficiency diet		
	Patient 1	Patient 2	Patient 3
Type of lesion			
Initial changes on tip: slight fissuring	38	49	14
Progression of initial change	45	13½	49
Early perlèche	77	56	49
Distinct perlèche	80	70	76
Balding of tongue	77	98	62
Dryness of tongue	54	67	48
Marked deficiency	122	101	94
Subjective changes			
Disturbance of taste	77	52	Exact onset not known
Difficulty in swallowing	77	98	
Burning of tongue and lips	80	52	

TABLE 11
CHANGES OF THE TONGUE IN THE PHASE OF VITAMIN REPLETION

	Days following vitamin therapy		
	Patient 1	Patient 2	Patient 3
Type of change			
Improvement of color (more pink)	4	4	3
Redevelopment of filiform papillae	4	4	7
Perlèche decreasing	3	—	2
Perlèche healed	22	4	22
Subjective changes			
Improvement of taste sensation	8	Not known*	15
Improvement of swallowing	—	4	—
Disappearance of burning of tongue		Not known	
Dryness of tongue disappeared	4	4	30

* Not definitely known because taste disturbances improved following discontinuation of Pteropterin administration

growth of the filiform papillae was noted in the central part of the tongue 7 days after vitamin repletion was started. Regrowth of the filiform papillae was noted on the tip and on the margins of the tongue by the twenty-second day of vitamin supplementation. At that time, the color of the tongue was normal. The observations cited continued to be approximately the same for the 33 days of supplementation of vitamins to the deficiency diet (the total duration of intake of the rice and fruit diet was 131 days). The patient then received a normal diet containing 68 gm. protein for 39 days, and the vitamin supplemen-

tation was continued as outlined in TABLE 4. In multiple observations made during this period of time, the color and moisture of the tongue were found to be normal. However, the shortening of the filiform papillae on the tip of the tongue persisted, while an increase in their length was noted in the center of the tongue after 2 weeks of full dietary intake.

The sequence of events noted in the depletion and repletion phases of Patient 3 is listed in TABLE 9.

TABLES 10 and 11 list appearance of the deficiency changes and the changes during vitamin therapy of the three patients.

Conclusions

Observations made in patients receiving a strict, controlled regimen of a deficient dietary intake have revealed that the development of deficiency changes of the tongue is slow and progresses gradually over a period of several weeks. Minimal objective changes were recognizable after 28 and 39 days of severe protein and vitamin restriction in Patients 1 and 2 while Patient 3, who was in subclinical avitaminosis before the study, showed these signs after 14 days of dietary restriction. The main findings were alterations of the normal pattern of the filiform papillae which started at first at the tip of the tongue and progressed slowly to other areas of the tongue mucosa. The decrease of the number of the filiform papillae was a gradual one which ultimately led to their complete disappearance. In contrast, the fungiform papillae were less affected by the deficiency state and became more prominent due to the decrease of the number of filiform papillae. In the phase of vitamin repletion, the changes occurred rather rapidly, and objective signs of improvement were noticeable within a few days of intravenous vitamin administration. The time interval needed for the recovery following the intramuscular administration of vitamins was only slightly longer. It was also shown that the deficiency changes could be abolished by adding vitamins without changing the intake of protein.

Patient 3 presented, prior to receiving the depletion diet, a condition that can be described as borderline between normal and beginning signs of depletion. The filiform papillae still covered the entire dorsum of the tongue, but they were shorter at the tip and were somewhat blunted. After the depletion diet was given for 2 weeks, the tongue presented a condition which resembled that seen after 4 weeks of depletion starting from a normal condition. Probably the tongue of Patient 3 can be described best as subclinical avitaminosis at the beginning of the study. Therefore, the time required for the full development of deficiency signs depends on the base line condition of the tongue.

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Part V. The Periodontal Tissues

SOME ASPECTS OF COLLAGEN FORMATION*

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Despite the fact that the deficiency state known as scurvy had been recognized since antiquity, and had been classically described by Walter (1746) as involving the healing of wounds as well as the maintenance of wounds that have already healed, it was not until 1919 that Aschoff and Koch and then Hojer in 1924 carried out detailed histological studies. These were soon followed by those of Wolbach and Howe (1926), which related the action of the accessory factor vitamin C to the formation of proper connective tissue, particularly of collagen. A number of investigations based on histological examination or tensile strength studies have established beyond question the relationship between ascorbic acid and collagen formation. Nevertheless, little is known concerning the exact mechanism by which ascorbic acid acts; both the cellular physiology and the biosynthetic mechanisms await clarification.

Two early theories have been the basis of much of the work attempting to elucidate the mechanism of the interaction between ascorbic acid and collagen fiber formation. Aschoff and Koch (1919) suggested that the defect in scurvy involves an inability to produce extracellular substance. Wolbach and Howe (1926) suggested that the secretion of intercellular ground substance and of collagenous precursor materials proceeds quite normally, but that some factor is lacking that normally causes gelling and fibrillation of a precursor in the extracellular material, and that this factor is not involved in the formation of fibroblasts. They found, from histological examination of scorbutic teeth and bones, an apparent accumulation of a fluid substance presumably secreted by the odontoblasts and osteoblasts that remained fluid due to the absence of a gelling factor. When vitamin C was administered, gelation occurred with such rapidity that they believed there had not been sufficient time for a new formation of collagen and suggested that "the failure of cells to produce intercellular substances in scorbutus is due to the absence of an agent common to all supporting tissues which is responsible for setting or gelling of a liquid product." Soon after the administration of vitamin C to depleted animals, an apparently homogeneous, amorphous substance that stained blue with Mallory's connective tissue stain formed around the cells. This was followed rapidly by the formation of reticulin fibers embedded in the amorphous material that Wolbach (1933) believed to be amorphous collagen.

Opposed to this concept of normal precursor formation in the absence of some

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fibrillating factor was that of Fish and Harris (1934) that the failure to heal is due to a disturbance of fibroblast metabolism with a consequent failure in collagen fiber formation Ham and Elliott (1938) supported this view and concluded that the primary defect in the scorbutic animal is an impairment of cellular secretion. Hojer's classic work (1924) also suggested that the defect involved in scurvy was associated with an impaired function of the connective tissue cell. The present investigation was aimed at testing certain aspects of these two hypotheses by chemical and histological methods.

Materials and Methods

Animals. Guinea pigs weighing 250 to 300 gm. and averaging about 275 gm were placed on a scorbutigenic diet that resulted in first signs of scurvy within 13 to 15 days, death usually occurred in 30 to 34 days. Normal control animals were kept on this diet, which was supplemented daily with 10 or 20 mg. of L-ascorbic acid given orally by dropper. After the animals had been maintained on the scorbutigenic diet for 7 days to ensure depletion of tissue and of blood ascorbic acid, they were prepared as follows. The hair of the intrascapular region was removed, the skin was cleaned with 70 per cent alcohol, and an incision 5 to 6 mm. long was made on either side of the mid-line. A polyvinyl sponge, prepared as described below, was inserted aseptically through each incision so that the sponge rested at a distance of 2 to 3 cm from the incision. The incision was then closed by means of a single suture.

Collection of tissue. At various intervals after implantation of the sponge, during which period the formation of connective tissue had proceeded as far as possible, the sponge was recovered by means of forceps through a small incision made indirectly above it. In most instances the removal was extremely simple but, when extensive collagen formation occurred, care was taken to avoid collecting any of the dense collagenous capsule that often surrounded the sponge.

The tissue-filled sponge was dried in acetone for 24 hours, minced finely, and re-extracted with acetone for an additional 24 hours The acetone-treated tissue was defatted with anhydrous ethyl ether, transferred to a 13 by 100 mm. Pyrex tube, and dried overnight at 108° C. in a vacuum oven.

Polyvinyl sponge implants. Polyvinyl Ivalon surgical sponge was used throughout The sponge, as obtained commercially, was allowed to dry in air until it was stiff enough to slice on an electrically driven food-slicing machine Sheets were cut about 3 mm. thick, and disks about 12 mm. in diameter were cut from these by means of a sharpened cork borer. Uniform disks weighing between 19 and 20 mg. were selected; these were washed repeatedly to remove free formaldehyde, distributed in tubes of distilled water, and sterilized at a steam pressure of 15 lb./sq. in. for 20 min. The wet sponge was implanted as described above.

Chemical methods; isolation of collagen. The collagen from the dried sponge was converted to gelatin by adding 3 ml. of water to each sample tube and autoclaving for 3 hours at a steam pressure of 25 lb./sq. in. The extract was transferred to a tared 13 by 100 mm. tube, and the residue was re-extracted with an additional 2 ml. of water by autoclaving, as before. The resulting

extracts were combined and evaporated to dryness in a current of air on a steam bath. The extract was dried to constant weight *in vacuo*.

Hydrolysis of autoclaved extract. The autoclave-extractable material was hydrolyzed in 6 *N* HCl in a sealed tube by heating at 150° C. for 3 hours. The hydrolyzate was neutralized with the theoretical amount of NaOH and diluted as desired for analysis.

Hydroxyproline determinations. These were carried out by the method of Neuman and Logan (1950), as modified by Martin and Axelrod (1953). The hydroxyproline value multiplied by 7.46 may be taken as a measure of the apparent collagen content. This would include fibrous collagen and precollagenous substances containing hydroxyproline. The results are expressed generally in micrograms of hydroxyproline in the total implant.

Histological methods. Sponges with the surrounding tissues intact were fixed in formalin and embedded in paraffin. Sections 6 to 7 μ in thickness were stained with hematoxylin-eosin, with Mallory stain for connective tissue, and by Laidlaw's (1929) and Gridley's (1951) methods for reticulin. Confirmation of the degree of scurvy was made by the classic histological bioassay technique as modified by Crampton (1947), which involves the sectioning of a hemisection of the mandible just anterior and parallel to the first molar, at right angles to the longitudinal axis of the mandible. The decalcified (5 per cent HNO_3) sections were prepared in the same manner as were the sponges and stained with either hematoxylin eosin or Wilder's silver stain.

Possible Accumulation of Collagen Precursor in Ascorbic Acid Deficiency

Collagen uniquely contains two amino acids, hydroxyproline and hydroxylysine, which cannot be incorporated directly into protein. Stetten and Schoenheimer (1943) have shown that when labeled proline is fed to animals it is incorporated into collagen as both proline and hydroxyproline. Stetten (1949) showed further that when labeled hydroxyproline was fed it was not used to any significant extent in the biosynthesis of collagen. She concluded that the hydroxyproline of collagen is derived from proline and that the conversion occurs after the proline has been incorporated into a peptide or larger molecule. A similar situation with respect to hydroxylysine has been found by Van Slyke and Sinex (1958) and by Piez and Likins (1957). It is interesting that Steward *et al.* (1958), studying the biosynthesis of a soluble proline-hydroxyproline-containing protein produced by actively growing carrot tissue cultures, have observed an almost identical relationship.

One explanation put forward for the apparent inability of an animal to utilize either hydroxyproline or hydroxylysine directly has been that a precursor protein is formed, which contains a high percentage of proline with little or no hydroxyproline. In subsequent biosynthetic steps the proline of such a possible precursor is partially oxidized to hydroxyproline. Robertson and Schwartz (1953) showed from a study of collagen biosynthesis in carageenan-induced granulomas in scorbutic guinea pigs that the administration of ascorbic acid leads to a much more rapid deposition of collagen than that encountered in natural repair. The scorbutic "carageenan" granuloma contains protein which, like collagen, is extractable by autoclaving, soluble in hot trichloroacetic acid

and precipitable with tannic acid. However, it contains little hydroxyproline. The amount of protein present was found to be almost equal to the amount of collagen in a normal granuloma and was rapidly replaced by collagen when ascorbic acid was administered. It was suggested that in ascorbic acid deficiency there is an accumulation of large amounts of a collagen precursor. However, when this protein was analyzed for its amino acid content it contained no excessively high concentration of proline or glycine and had an amino acid pattern markedly different from that of collagen.

Gould and Woessner (1957), in a study of collagen biosynthesis in healing skin wounds in normal and scorbutic guinea pigs, found that ascorbic acid-depleted animals given ascorbic acid at the time of wounding and animals on a normal diet both produce the bulk of hydroxyproline between the sixth and eighth day after wounding, whereas similar animals maintained on a scorbutigenic diet produce no hydroxyproline. However, upon the administration of ascorbic acid ten to twelve days after wounding, the animals maintained on the scorbutigenic diet produce relatively large amounts of collagen within forty-eight hours This rapid formation of hydroxyproline suggested that a precursor, of the sort suggested by Robertson and Schwartz (1953), might be involved. A study of the amino acid changes in scorbutic granulation tissue and in implanted sponges after the administration of ascorbic acid indicated a sharp drop in the noncollagenous proline content and a concomitant increase in hydroxyproline. This led to the postulation of a presumptive proline-rich collagen precursor that accumulates with the deprivation of ascorbic acid. After the administration of the vitamin, this protein pool might be converted to a more immediate collagen precursor, perhaps tropocollagen, rich in hydroxyproline It was postulated that the biochemical defect in scurvy is the inability to carry out the hydroxylation of proline to hydroxyproline and, presumably, of lysine to hydroxylysine.

Robertson *et al.* (1959) reinvestigated this problem and isolated proline and hydroxyproline from carageenan granulomas of normal and of scorbutic guinea pigs recovering from ascorbic acid deficiency after the administration of labeled proline. Their results suggest that most of the collagen is synthesized completely during recovery and does not arise from a preformed proline-rich precursor. They believe that the amino acids enter the collagen molecule as separate entities and that there probably exists an "active hydroxyproline" pool derived but distinct from the "active proline" pool, in ascorbic acid deficiency the conversion of the active proline to active hydroxyproline is diminished.

To test the hypothesis that in scurvy a proline-rich precursor accumulates, attempts were made to isolate such precursor material from the granulation tissue of animals and from subcutaneously implanted polyvinyl sponges in guinea pigs deprived of ascorbic acid. Analysis of a variety of extracts yielded no fraction that was unusually rich in proline or glycine. Analysis of various extracts obtained by starch zone electrophoresis also failed to yield positive evidence of such a precursor.

Attempts to Remove Precursor Material by Repeated Flushing of Implanted Sponges

Sponges were implanted after a preliminary depletion period. The animals, in two groups, were maintained on the scorbutigenic diet for 12 days after im-

plantation. In one group, from the eighth to the twelfth day one sponge in each animal was flushed several times daily with several milliliters of 0.95 per cent sterile saline, while the control sponge was left intact. The purpose was to wash out, if possible, any accumulated precursor. Ascorbic acid (20 mg.) was administered for 4 days with no further flushing. In a second group the treatment was the same except that the flushing was carried out only during the period when ascorbic acid was being administered. From the data pre-

TABLE 1
EFFECT ON COLLAGEN FORMATION OF REPEATED SALINE FLUSHING OF IMPLANTED POLYVINYL SPONGES

Treatment	No of animals	Hydroxyproline formed (μg)	
		Treated sponges	Control sponges
Scorbutigenic diet 12 days, flushed daily eighth to twelfth day; then 20 mg ascorbic acid for 4 days, no flushing	14	136	167
Scorbutigenic diet 12 days; then 20 mg. ascorbic acid daily, flushed daily, for 4 days	11	141	144

TABLE 2
COLLAGEN SYNTHESIS IN NORMAL, MOVED, AND REPLACED SUBCUTANEOUSLY IMPLANTED POLYVINYL SPONGES

Sponge	Preliminary treatment	No of animals	Days ascorbic acid administered*	Hydroxyproline formed (μg)
Control	12 days scorbutigenic diet	10	5	168
Control	12 days scorbutigenic diet	4	8	219
Mock removal	12 days scorbutigenic diet	10	5	132
Replaced by new	12 days scorbutigenic diet	10	5	112
Replaced by new	12 days scorbutigenic diet	4	8	185
Moved	12 days scorbutigenic diet	7	5	42
Moved	12 days scorbutigenic diet	4	8	169
New	none	9	5	5
New	none	5	8	13

* Twenty mg. ascorbic acid per day.

sented in TABLE 1 it appears that such treatment does not remove any essential precursor material.

Transfer of Sponges

A series of experiments was carried out in which sponges were allowed to organize in animals maintained on the scorbutigenic diet for 12 days. At the end of this time the sponges were very carefully removed from the delicate capsules that form around them and were implanted in new subcutaneous areas in the same animal. At the same time a fresh sponge was placed in the first area. The animals were then given 20 mg. ascorbic acid daily for from 5 to 8 days, and then the sponges were analyzed for hydroxyproline. From the data presented in TABLE 2 it is quite evident that the sponges replacing previ-

ously organized sponges promptly formed rather large amounts of collagen; whereas the transferred, organized sponges produced only small amounts of collagen in the first 5 days, but thereafter produced larger amounts. It appears that the capsule and its blood vessel supply are of primary significance. Once the newer sponge develops these, it can form collagen, before this, even though it is full of cells, mucopolysaccharide, and protein, it cannot form collagen promptly. Histological examination (FIGURE 1*A* and *B*) indicates that collagen

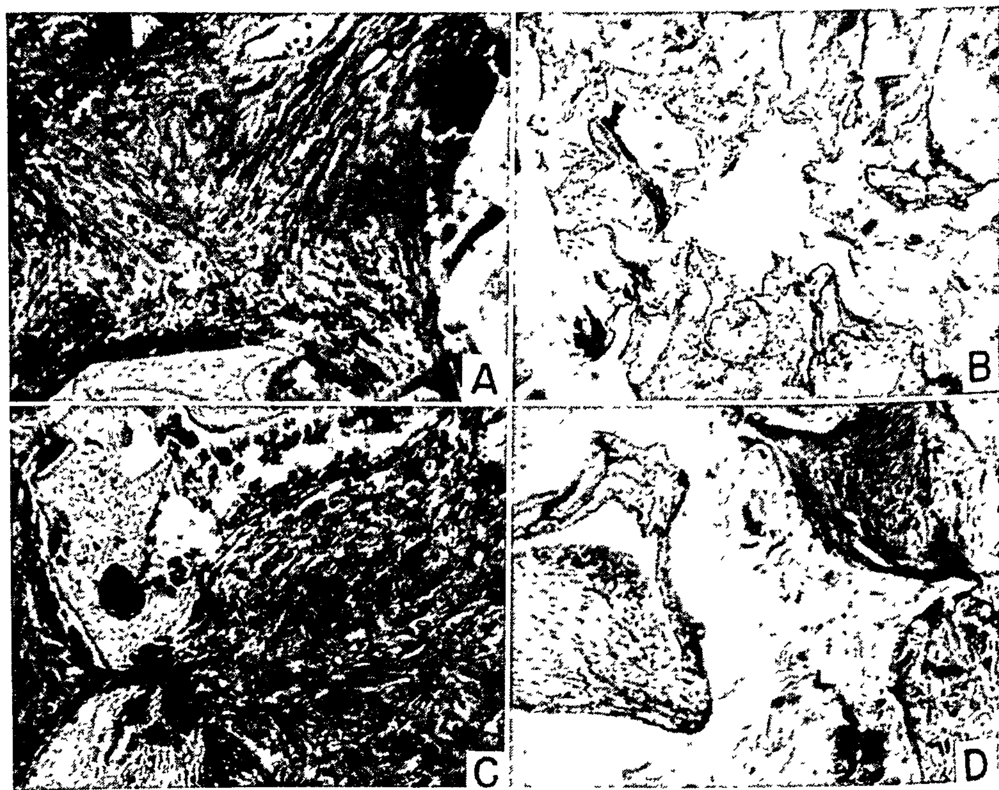


FIGURE 1 (*A*) Section of a sponge inserted where another sponge had been implanted and organized during 12 days of a scorbutigenic diet After insertion, 20 mg ascorbic acid was administered for 5 days (*B*) Original sponge implanted in new subcutaneous site, sectioned after 5 days' treatment with 20 mg ascorbic acid daily (*C*) Section through a sponge injected with hyaluronic acid several times daily during 5-day recovery period. (*D*) Sponge injected several times daily with hyaluronidase during 5-day recovery period

formation proceeds by a migration of material from the capsule. This does not mean that some precursor material is not present in the sponge itself.

Labeled Proline Studies to Test the Hypothesis of Precursor Accumulation

An experiment analogous to that performed by Robertson *et al.*, who used carageenan granuloma, was carried out with implanted polyvinyl sponges. A group of guinea pigs deprived of ascorbic acid was given proline-C^{14} during the days just after wounding and maintained on the scorbutigenic diet for 12 days. Another group was maintained on the scorbutigenic diet for 12 days after wounding, at which time they received both proline-C^{14} and ascorbic

acid. The results (FIGURE 2) of analyses of collagen proline and hydroxyproline from both groups gave no evidence of any accumulation of labeled hydroxyproline in those animals treated with labeled proline during the period between wounding and ascorbic acid administration. Actually, large amounts of proline were converted to hydroxyproline in the animals simultaneously receiving proline and ascorbic acid. The specific activities of proline and hydroxyproline in the newly formed collagen declined rapidly in the group that had received proline-C^{14} prior to the administration of ascorbic acid, whereas it remained substantially constant in the group that received proline-C^{14} together with ascorbic acid. In agreement with the findings of Robertson *et al.* and Green

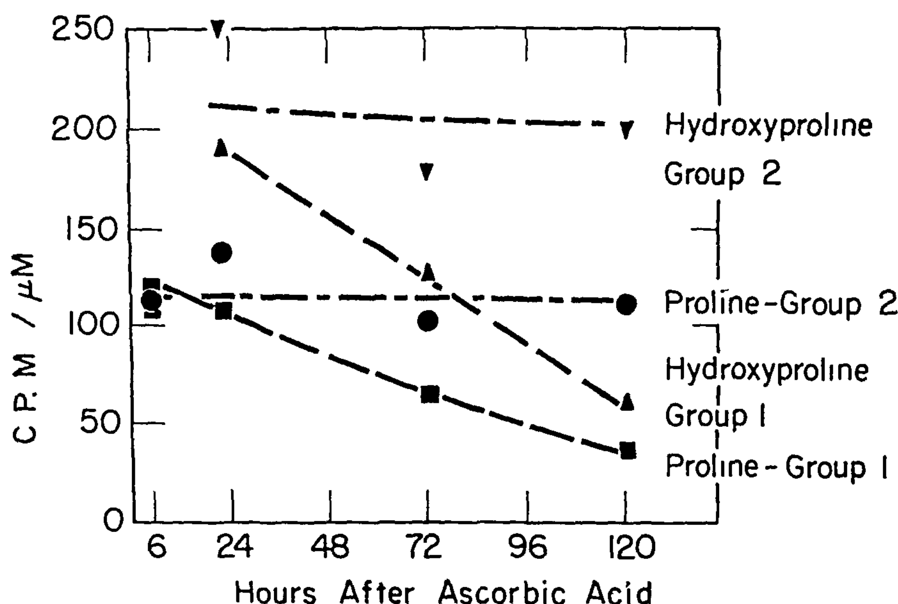


FIGURE 2. Incorporation of proline-C^{14} into newly formed collagen. Animals maintained on scorbutigenic diet for 5 days prior to and 10 days after sponge implant. Ascorbic acid 20 mg administered daily from the tenth postoperative (0 time on diagram) day onward. Sponges removed at 6, 24, 72, and 120 hours after commencement of ascorbic acid treatment. Group 1 animals each received 12 μc of L-proline-C^{14} in 3 equal doses given 7 or 4 days or 1 day prior to first dose of ascorbic acid. Group 2 animals received same dose of labeled proline at 0 time, 24 hours, and 72 hours after first dose of ascorbic acid.

and Lowther (1959), the ratio of the specific activity of hydroxyproline to that of proline was greater than 1 in all animals.

In spite of the fact that hydroxyproline is derived from proline and that the conversion appears to occur after the incorporation of the proline into some activated form—a peptide or larger molecule—only presumptive evidence for the occurrence of any such precursors has been presented.

Role of Ascorbic Acid in the Maintenance of Collagen

Although there is little question that a relationship exists between ascorbic acid and collagen production, the relationship between ascorbic acid and the maintenance of collagen is not very clear. On the basis of experiments with labeled amino acid incorporated into collagen, Neuberger *et al.* (1951) and Robertson (1952) showed that collagen is metabolically inert compared to most

other proteins. However, data do exist indicating that collagen is not completely inert, but that there is a slow synthesis and degradation even in adult skin and tendon. In certain tissues, such as liver, bone, and periodontal membrane, the process can be relatively rapid, and in some systems the disappearance of collagen can proceed at an extremely rapid rate. Harkness and Moralee (1955) report the half life of the collagen in the involuting uterus as 1 to 2 days; in recovery from hepatic necrosis, described histologically by Cameron and Karunaratne (1936) and measured chemically by Morrione (1949), it was found to be about 10 days, Jackson (1957) reports it as 5 to 6 days in the carageenan granuloma; Noble *et al.* (1958) report it to be about 30 days in subcutaneously implanted polyvinyl sponges in the rat. Our own studies indicate that, upon withdrawal of ascorbic acid from guinea pigs bearing implanted polyvinyl sponges, the half life of the newly formed collagen is also about 30 days.

Some of the earliest reports of scurvy contain statements to the effect that wounds that had been healed for years would break down if a person became scorbutic (Walter, 1769). Hunt (1941) studied the effect on healed wounds of the withdrawal of vitamin C, and his conclusions, based on observation of a few animals, were that mature collagen in scars may retrogress if ascorbic acid is withdrawn from the diet for prolonged periods and that the new collagen had reverted to an argyrophilic precollagenous state very different from that found in normal control wounds. Pirani and Levenson (1953) also demonstrated that ascorbic acid is necessary for both the healing of wounds and the maintenance of the collagen that had formed They subjected guinea pigs to laparotomy and then allowed the wounds to heal for from 2 to 6 weeks. Some of the animals were then deprived of ascorbic acid, while pair-fed controls received ascorbic acid. About three weeks after the withdrawal of ascorbic acid, changes were observed in the wounds of many of the animals on the deficient diet. The connective tissue was loose, and numerous fibroblasts and immature mesenchymal cells were present; there were numerous capillaries, many of which were defective, and several areas of hemorrhage. The changes observed were of the same type as those occurring in healing wounds in scorbutic animals. However, Elster (1950) and Robertson (1952) found that in chronic scurvy the amount of collagen destroyed must be small or the degradation in structure must be slight Robertson concluded from his studies that ascorbic acid is not essential for the maintenance of preformed collagen and that possibly only certain recently formed collagen requires ascorbic acid for maintenance.

In the present investigation, polyvinyl sponges were implanted subcutaneously in ascorbic acid–depleted and in normal guinea pigs, and new fibrous tissue was permitted to accumulate. After varying periods of collagen formation the animals were placed on a scorbutigenic diet and the rate of collagen resorption from the sponges was determined chemically (TABLE 3) and studied histologically (FIGURE 3*A* to *F*). It was found that almost directly after the withdrawal of ascorbic acid there was complete disappearance of salt-soluble collagen and that this was followed by the slower disappearance of insoluble collagen. The rate of resorption was greater from sponges with newly formed collagen than from those implanted for a prolonged period, but the latter also

showed considerable resorption. When sponges were implanted in animals that had not been depleted, there was considerably less resorption. This last fact is difficult to explain since, even though the animals had been depleted, once the sponges were implanted ascorbic acid was administered for as long as 30 days before the animals were placed on the scorbutigenic diet. Histological examination, however, suggests that the difference may involve impaired blood vessel formation (FIGURE 3*E* and *F*) during the first few days of organization of the implant, during which time the animal has not been resaturated with the vitamin.

The mechanisms involved in collagen resorption are little, if at all, understood. It is not even clear whether the process involves collagenolysis by specific or nonspecific enzymes or merely removal of the particulate material by phagocytosis. Hunt (1941) suggested that scar collagen was converted to a precollagenous state after the withdrawal of ascorbic acid.

TABLE 3
RESORPTION OF NEWLY FORMED COLLAGEN AFTER WITHDRAWAL OF ASCORBIC ACID*

Ascorbic acid treatment before withdrawal	Hydroxyproline (μg) Days after withdrawal of ascorbic acid		
	0	7	21
(1) 20 mg daily for 12 days	185	106	36
(2) 20 mg daily for 28 days	173	71	59
(3) 20 mg daily for 28 days (no depletion before wounding)*	157	169	125
(4) 20 mg daily for 59 days	288	340	160 (33 days)

* All sponges except those in treatment 3 were implanted after a 6-day depletion period

The Role of Hyaluronidase in Collagen Formation

In cases of scurvy the accumulation of apparently depolymerized ground substance suggested by Gersh and Catchpole (1949) is in accord with the findings of several investigators who propose that the depolymerization of the ground substance is the result of hyaluronidase action; they also propose that, under normal conditions, ascorbic acid or its degradation products inhibit the enzyme, but that in its absence the ground substance is depolymerized and the formation of collagen is prevented Reppert *et al.* (1951) claimed that ascorbic acid inhibits hyaluronidase *in vitro*. However, Patterson and Cole (1952) found that ascorbic acid itself did not inhibit the enzyme, but that dehydroascorbic acid did, and that the further oxidation products of dehydroascorbic acid are even more strongly inhibiting. They suggested that ascorbic acid serves as a reservoir where compounds that control the hyaluronidase system are formed. The accumulation of hyaluronic acid in scurvy observed by Robertson and Hinds (1956) and by Slack (1958) suggests that the absence of ascorbic acid cause a decrease in hyaluronidase activity that would result in the inability of the scorbutic animal to remove the hyaluronic acid from immature fibrils and thus would prevent their maturation.

Scorbutic wounds appear to be composed of thick, chaotically arranged,

precollagenous fibers that suggest the accumulation of a mucopolysaccharide sheath around a precollagen core. Supposedly, the mucopolysaccharide interferes with the subsequent maturation of the collagen. In a study of granulomatous repair tissue in normal and scorbutic guinea pigs produced by the subcutaneous injection of carageenan Robertson and Hinds 1956) found that the collagen-poor tissue that accumulated under scorbutigenic conditions contained about five times as much mucopolysaccharide as the repair tissue formed

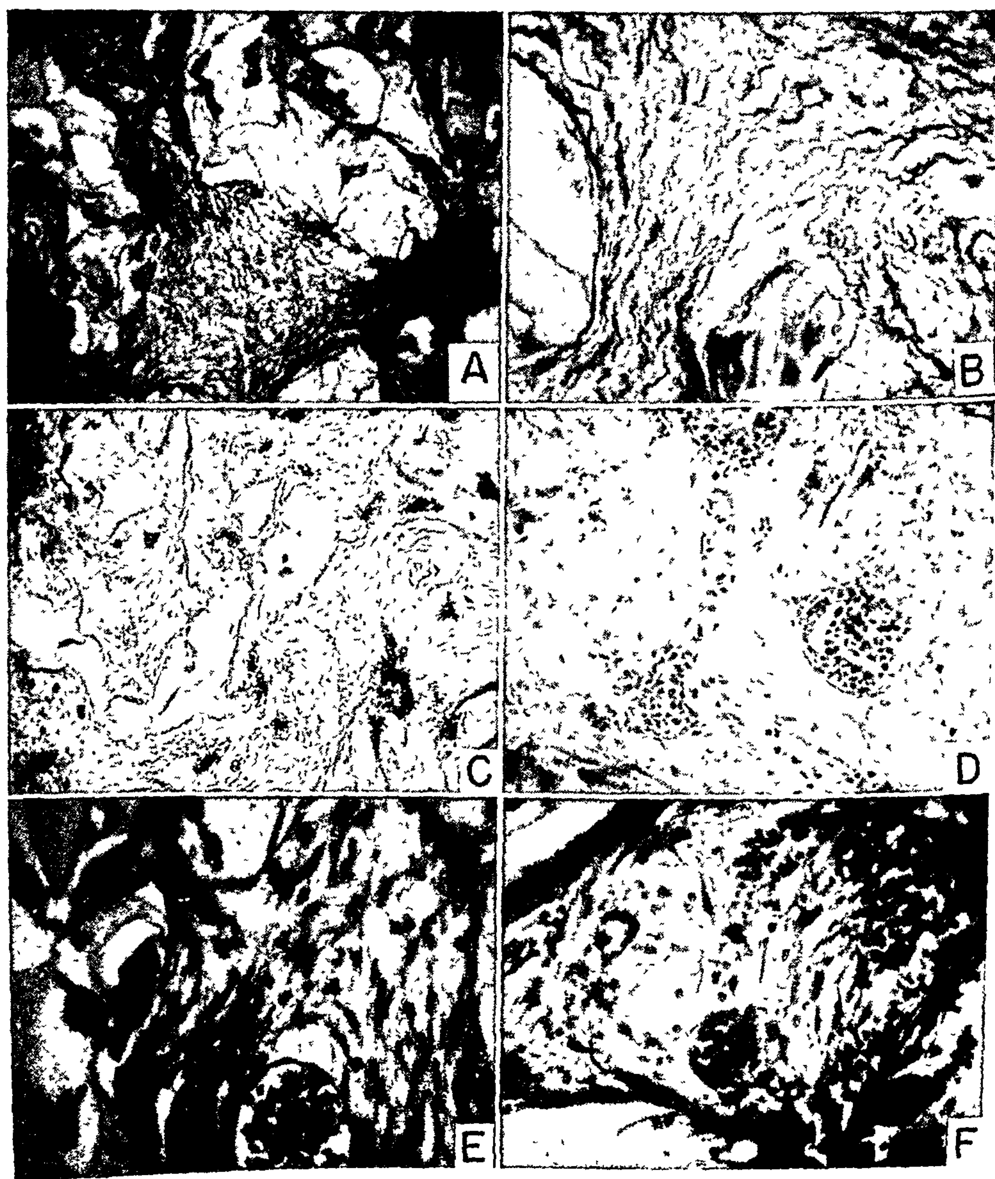


FIGURE 3. (*A*) Section through a 30-day-old sponge maintained in an animal fed a diet supplemented with 20 mg. ascorbic acid daily, showing dense fiber formation (*B*) Higher magnification of same section. (*C*) Effect of withdrawal of ascorbic acid for 21 days after 30-day period of collagen formation (*D*) Higher magnification of same section (*E*) Sponge treated as in *A*, stained with hematoxylin-eosin, intact blood vessel walls (*F*) Section comparable to that in *C*; resorption of collagen from blood vessel wall that breaks open

under normal conditions. Fractionation of the tissue indicated that the polysaccharide that accumulates may be hyaluronic acid. Slack (1957, 1958) supported the findings of Robertson and Hinds (1956) by demonstrating that in scorbutic carageenan-induced granulomatous tissue there is a greatly increased accumulation of polysaccharide that, in fact, is due to an increase in hyaluronic acid or in compounds with very similar properties. Penney and Balfour (1949) found that the healing wounds of scorbutic guinea pigs failed to produce acid mucopolysaccharides, but that after the administration of even small amounts of ascorbic acid a large amount of mucopolysaccharide, either chondroitin sulfate or hyaluronic acid, was promptly produced. According to these investigators, fiber formation involves first the deposition of mucopolysaccharide around the fibroblasts.

Gross *et al.* (1952) were able to convert purified soluble collagen to normal fibers *in vitro* with the use of mucroprotein, serum glycoprotein, hyaluronic

TABLE 4
EFFECT OF VARIOUS SUBSTANCES ON HYDROXYPROLINE (COLLAGEN) FORMATION IN SUBCUTANEOUS POLYVINYL SPONGES*

	No of animals	Hydroxyproline (μg)	
		Injected sponge	Control sponge
Hyaluronidase†	9	199	180
Chondroitin-SO_4‡	4	165	151
Hyaluronidase inhibitor§	8	210	175
Carageenan‡	5	148	180
Hyaluronic acid‡	4	104	141

* All animals received 10 mg ascorbic acid per day for 4 days.
† Injections 10 mg per day in 5 doses (250 turbidity units/mg).
‡ Injections 2 mg per day in 5 doses
§ Injections 1 mg. per day in 5 doses (nitrated hyaluronic acid).

acid, or chondroitin sulfate, but the effect was found to be nonspecific. Similar results were obtained by Jackson and Randall (1955). Gross (1956) found that merely altering the ionic strength of the solution induces fiber formation if a solution is kept at 37° C., indicating that although mucopolysaccharides may be involved in the fibrillation of soluble collagen they are not essential. In studies of the influence of various electrolytes and nonelectrolytes on *in vitro* fibril formation, Gross and Kirk (1958) found that chondroitin sulfate, hyaluronic acid, and acid glycoprotein of serum had no effect on the rate or extent of fibril formation and that ascorbic acid actually had an inhibitory effect.

In the present study, sponges were implanted bilaterally and subcutaneously and the animals were maintained on the scorbutigenic diet for 12 days. Ascorbic acid was administered for the next 4 days; during this period one of the sponges in each animal was injected several times daily with either hyaluronidase, hyaluronidase inhibitor, hyaluronic acid, chondroitin sulfate, or carageenan. The control sponge was injected with sterile saline The results given in TABLE 4 indicate that the total collagen formed is not appreciably different in the control and in the experimental sponges. Histological examination of the

hyaluronidase-treated sponges (FIGURE 1*D*) reveals fibers that are not as sharply defined as those from normal sponges and considerably less so than those from hyaluronic acid–treated sponges (FIGURE 1*C*).

There is no proof that mucopolysaccharides are specifically involved in the formation of collagen fibrils; they may have to do with other functions of these tissues and relatively little, if anything, to do with the formation of collagen fibrils. However, it must be kept in mind that they may not play an active role in fibrillation *in vivo* but may play an important passive role.

The Role of Ascorbic Acid in Cultured Embryonic Tissue Development

Woessner and Gould (1957) made quantitative studies of collagen formation by chick embryo lung tissue grown in media deficient in, or completely lacking,



FIGURE 4 Section of an embryonic tooth germ showing the odontoblast layer The tooth was implanted in the anterior chamber of the eye of a guinea pig subsequently maintained on a scorbutigenic diet for 35 days *OD*, the odontoblast layer

ascorbic acid. They used the roller tube technique. Cell growth and collagen formation were found to proceed quite well in cultures lacking ascorbic acid. The addition of ascorbic acid to whole embryo extract media, dialyzed media, or synthetic media had no appreciable effect on total collagen formation.

The embryonic tooth germ was selected to determine whether embryonic tissue had peculiar requirements with respect to ascorbic acid. It was expected that the maintenance of the odontoblasts and the formation of dentin would be an exquisitely sensitive system. The earlier unpublished observations of Gould, Goldman, and Woessner of such tooth germs implanted in the anterior chamber of the guinea pig eye have been expanded. It has been found that, when implanted in depleted guinea pigs and maintained for as long as 5 weeks, at which time the host died of scurvy, such tooth germs showed quite normal odontoblast structure (FIGURE 4). Furthermore, embryonic bone implanted under similar conditions also showed normal development. It would appear that embryonic tissue may not require an external source of ascorbic acid for its mesenchymal development.

Summary and Conclusions

The results of experiments designed to test the hypothesis that impaired collagen formation in ascorbic acid deficiency results in the accumulation of an unhydroxylated precursor are presented. None of the results of isolation procedures, transplantation experiments, extensive perfusion of implanted polyvinyl sponges, and studies with labeled C^{14}-proline could substantiate the presumptive evidence for such a precursor.

Other experiments, aimed at demonstrating a causal interaction of components of the ground substance on collagen production do not suggest that hyaluronic acid, hyaluronidase, hyaluronidase inhibitor, or chondroitin sulfate have a marked effect on collagen formation.

Evidence is presented to substantiate the idea that ascorbic acid is essential for the maintenance of newly formed collagen and of blood vessel walls.

Preliminary experiments are described in which embryonic guinea pig tooth germs were implanted in the anterior chamber of the eyes of animals maintained until death on an ascorbic acid–free diet. The normal growth and development of such teeth suggested that embryonic mesenchymal tissue does not require ascorbic acid.

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A THEORY OF CONNECTIVE TISSUE BEHAVIOR: ITS IMPLICATIONS IN PERIODONTAL DISEASE*

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During the past ten years we have studied the structure and behavior of many kinds of mammalian connective tissue, using morphological, chemical, and physicochemical methods. From this work certain unifying concepts have emerged In this paper these are reviewed, and their implications in periodontal disease are considered.

Morphology and Histochemistry of Connective Tissue

Connective tissues permeate the entire organism, forming a continuum that is the immediate environment of the cells. Despite the specialized characters of the different kinds of connective tissue, there are certain important common features. In all connective tissues two principal constituents are present: cells, and the extracellular matrix that is largely derived from cells The extracellular part is formed of fibers, chiefly collagenous, and also of varying amounts of elastin and reticulin, depending upon the tissue. The fibers are embedded in an optically homogeneous matrix or ground substance that includes water and electrolytes.

It has been shown by chemical and histochemical methods that carbohydrate-protein complexes form an important part of the extracellular ground substance.[1-3] With the optical microscope the ground substance does not have a discernibly organized structure. However, when certain dyes and histochemical reagents are applied to tissue sections, the structure can be demonstrated. This is due to its anionic character and to the presence of carbohydrate-containing substances. Metachromasia is observed when some connective tissues are stained with such cationic dyes as toluidine blue. This is believed to indicate the presence of electronegative macromolecular aggregates which, in the case of connective tissues, are sulfonated.[4-5] Bound carbohydrate in tissues may be visualized by subjecting sections to mild oxidation with periodic acid followed by the Schiff reagent. The reaction principally identifies aldehyde groups derived from the oxidation of carbohydrate-containing components of the ground substance [6-9] When the method is properly controlled by enzyme studies and careful fixation, such as can be achieved with freeze-drying, it is specific for certain glycoproteins or heteropolysaccharides.[10] The periodic acid-Schiff (paS) reaction has been useful for making a crude characterization of the ground substance and for studying its biological reactivity.

The intensity of the paS reaction varies, depending upon the tissue and its state. For example, loose watery tissues such as the pulp of a tooth or very young skin stain only lightly, whereas gingival tissues and cartilage stain progressively more intensely. Gersh and Catchpole[1] suggested that there was a

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correspondence between staining intensity and concentration of reacting groups. They attributed certain degrees of aggregation to the connective tissue ground substance, these degrees ranging from low for synovial fluid and umbilical cord jelly to high for cartilage, bone, dentin, and cementum. Where the state of aggregation was considered low, the concentration of groups that could be oxidized by periodic acid also was deemed low, hence the weak tinctorial reaction. Higher levels of aggregation was associated with a greater density of reactive groups and a more intense reaction This interpretation was qualified to explain the virtual failure of mature bone and dentin to stain.[2,10-14] It was considered that in such tissues the closely compacted organization reflected a high order of interaction and cross-linkage of reactive groups; hence these were no longer available for periodic acid oxidation, and the Schiff reactive material also was of a lower order of concentration. Changes in the aggregation or cross-linking of such tissues theoretically would enhance their reactivity with the paS reagent, indeed, this was shown to be the case[11-14]

Periodontal tissues. In order to describe the microscopic distribution of the glycoproteins of the ground substance in some representative tissues, we shall choose as examples the free gingivae, the periodontal membrane, alveolar bone, and cementum.

In freeze-dried preparations of gingiva (FIGURE 1) an irregular demarcation separating the epithelium from the underlying connective tissue is seen. This undulating line stains intensely with the periodic acid method. It is called the basement membrane. On closer examination, this structure is seen to consist of a zone of ground substance containing embedded reticular fibers. The organization and staining of the basement membrane serve as a crude criterion of the general state of the contiguous connective tissue In the subepithelial layer below the basement membrane lie the connective tissue cells and the collagenous, reticular, and elastic fibers embedded in the ground substance. This layer reacts to the stain with a pink, red, or magenta color. This pattern of ground substance-fiber contiguity was observed in other connective tissues, such as those of pulp, skin, monkey sex skin (FIGURE 1), and avian comb In the connective tissue meshwork the small blood vessels are delineated by a deeply stained peripheral basement membrane. Fibrillar components of basement membrane may be demonstrated most clearly with a silver stain in conjunction with the paS method.

With respect to the immediate periodontium formed by periodontal membrane, bone, and cementum, it is now becoming possible to study these tissues in a condition that is relatively free of artifacts. John Riedlinger, who is working in this laboratory, is perfecting methods for freeze-dry fixation, embedding, and sectioning of the periodontal tissues without decalcifying This will open up the possibility of much work in a field heretofore neglected because of technical difficulties

Some of the preliminary observations made by these methods are the following. Periodontal membrane stained with the paS reagents appears as a fibrous layer embedded in an intensely red-stained matrix of ground substance. Large fibroblasts are dispersed throughout the tissue. Where the fibrous connective

tissue merges on the opposing sides with the alveolar bone or the cementum, the cellular cytoplasm is seen to contain readily resolvable granules of glycoprotein that may be precursors of the ground substance. The narrow margins of bone and of cementum contiguous with the periodontal membrane are deeply stained with paS. Under normal conditions, the bone and cementum beyond this give only a very faint pink reaction

Submicroscopic organization of connective tissue. When connective tissues are viewed with the electron microscope, periodic structures are noted in collagen and reticular fibers, but not in the elastic ones. If special methods of fixation and staining are followed, a degree of organization is seen in the ground sub-

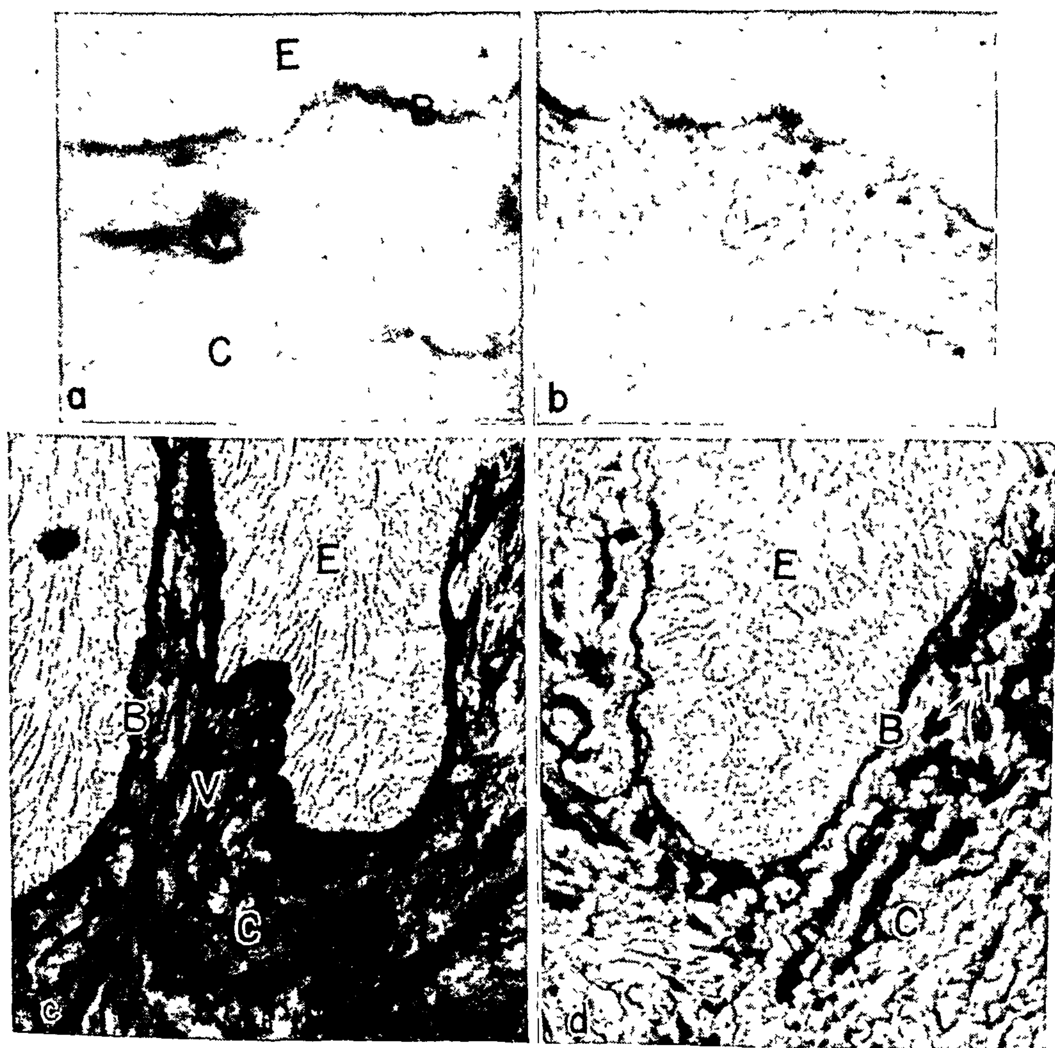


FIGURE 1 Sections of connective tissue, 6μ, fixed by freeze-drying and stained by the paS method after alcohol denaturation. *B*, basement membrane, *C*, connective tissue, *E*, epithelium, *I*, cells of inflammation, *V*, blood vessel (*a*) Ground substance of prepuberal monkey sex skin before estrogen. ×770 (*b*) Ground substance of prepuberal monkey sex skin after estrogen (6000 R U) ×770 (*c*) Ground substance of normal human gingiva. ×620 (*d*) Ground substance of gingiva showing pregnancy gingivitis. Ice crystals, representing increased hydration, are prominent. ×620

stance, as described by Bondareff.[15] This substance appears as a system of submicroscopic vacuoles enclosed by denser walls. The vacuoles of the ground substance of fetal rat tendon have dimensions of 500 to 2000 Å. The electron-dense walls of the vacuoles are believed to be rich in protein, and the vacuoles themselves are thought to contain chiefly water. These two morphologic phases correspond to the colloid-rich, water-poor phase and the water-rich, colloid-poor phase that we had postulated earlier on physicochemical grounds.[16]

Further confirmation of this heterogeneous organization in connective tissue was obtained from the work of Chase[17] and of Dennis[18] based on the localization of intravitally injected ferrocyanide ion in connective tissue. Sections of connective tissue were treated with ferric chloride in anhydrous alcohol, inducing the Prussian blue reaction. The sites of precipitated Prussian blue corresponded to the vacuoles described by Bondareff.[15] It appears that the walls of the vacuoles are coextensive with the collagen fibers. Thus on morphologic grounds there is evidence of a close relation of fibers and extrafibrillar ground substance. Indeed, Schwarz[19] and Grassmann *et al.*[20] have used periodic acid oxidation coupled with a silver method to demonstrate presumptive carbohydrate-containing material in association with the internal fine structure of collagen fibers.

Chemical and Physicochemical Properties of Connective Tissues

The morphologic evidence for the existence of two phases in connective tissue is augmented by the most elementary chemical evidence. At least two fractions have been separated from dermis, a fraction that is soluble in neutral salt (0.15 *M* NaCl in phosphate buffer) and an insoluble residue.* These two fractions have been extensively studied by Gross,[21] Jackson,[22] Orekhovitch and Shpikiter,[23] and others. Also, freeze-dried tissue sections of skin, gingiva, primate sex skin, bone, cartilage, and other connective tissues contain water, or neutral salt-soluble glycoproteins, and insoluble glycoproteins[1-2,24-25] The "soluble collagen" has been equated with tropocollagen or with procollagen, subunits or precursors of the mature collagen. The "soluble collagen" actually contains a heterogeneous group of substances that includes bound hydroxyproline, sulfate, and glycoproteins, representing components of both the ground substance and the fibrous constituents. Soluble collagen is therefore derived from a part of the water-rich, colloid-poor phase of connective tissues. The insoluble residue is identified with the protein-rich, water-poor phase.

Chemical characterization of connective tissues depends on the isolation of proteins, including collagen and glycoproteins, mucopolysaccharides, lipids, and other highly complex aggregates. Independently of the detailed characterization of the various fractions, certain physicochemical properties of this organic matrix may be described. The components are conceived of as interacting through various kinds of chemical bonding, as yet undefined, to form a colloid macromolecule or coacervate[26-27] that acquires emergent properties.

The presence of such acid substances as sialic acid, chondroitin sulfate, and hyaluronic acid as integral components of the carbohydrate-protein complexes,

* This fraction has been fragmented further by extraction with more drastic procedures, such as with the use of acetic acid, buffered citrate, and more concentrated salt solution.

as well as a preponderance of carboxyl groups on the protein itself, confers on the connective tissues a negative charge at physiological *p*H. Thus the tissue exhibits the behavior of an immobile charged colloidal polyelectrolyte[3,16,25,27-30] whose composition varies from tissue to tissue. For example, the ratio of collagen, determined as hydroxyproline, to hexosamine for cartilage, skin, and tendon (per cent dry weight) are 27/7.98 (2.8), 65/0.53 (12.2) and 0.78/0.26 (30), respectively. The sulfate concentrations (millimoles per kilogram water) are 120 to 140, 0.8, and 5.0, respectively. These analytic results have significant implications for the understanding of the internal linkages of the coacervate and its physicochemical behavior,[27] as will be considered below.

We have adapted an electrochemical method to the *in vivo* study of the charge density in many kinds of mammalian connective tissue.[16,25,27-30] This has enabled us to measure the negative charge density in bone, cartilage, gingiva, guinea pig pubic symphysis, monkey sex skin, tendon, and avian comb. Some unique characteristics of various connective tissues and of their reactions in physiological and pathological states are revealed by these studies.

Only a brief description of the method can be given here; more emphasis will be placed on the results and their implications. The method stems from the Donnan theory of membrane potentials in systems consisting of nondiffusible and diffusible ions[31] and from Henderson's theory of liquid junction potentials.[32] The theory was modified by Meyer and Bernfeld[33] and by Teorell[34] for the study of biological colloids. We have adapted it to the investigation of connective tissue. The method also has been applied to the study of epidermal[35,36] and mucosal surfaces and to the *in vitro* study of the fibrous protein surface of wool.[37]

The operation involves the use of a potentiometer and calomel-mercury half cells for the measurement of an electric potential (E_1) between a reference area* and a liquid-tissue boundary at some experimental site (FIGURE 2). KCl-agar junctions are used to make the tissue contacts. First, buffered (*p*H 7.4) isotonic sodium chloride is applied at both junctions (the solution is applied at the experimental site through small cotton pellet tips). Usually there is a small potential difference. Then a one-tenth dilution of sodium chloride is substituted at the experimental site and a potential E_2 is measured. The difference, $E_2 - E_1$, is called the dilution potential (E_d).

In an aqueous solution the theoretical potential at a boundary between 0.15 *M* NaCl and 0.015 *M* NaCl at 37° C. is −12.3 mv. However, this potential is altered where an immobile charged colloid is present. At physiological *p*H where the negative charge density is high, as in the case of cartilage or osteochondral tissue, the values may be around +20 mv. Values for skin are about 0 mv, tendon gives potentials of about −6 mv, and synovial fluid, which resembles an aqueous medium, gives values of about −12 mv (TABLE 1). To generalize: at physiological *p*H dense tissues give high positive potentials and loose tissues give negative values approaching that observed in water. From these potential values the density of immobile negative charge (X) in tissues has been calculated according to the equation $E_d = -12.3 + 206\,X$.

* In animal work the shaved tail, ear, or leg serves as the fixed reference, in work with human beings, a finger is used

The colloidal charge density is expressed in equivalents per kilogram of water and corresponds to the base binding capacity of the tissue. When the charge density is measured at different pH levels, in effect, an *in vivo* titration curve of the tissue colloid is established.

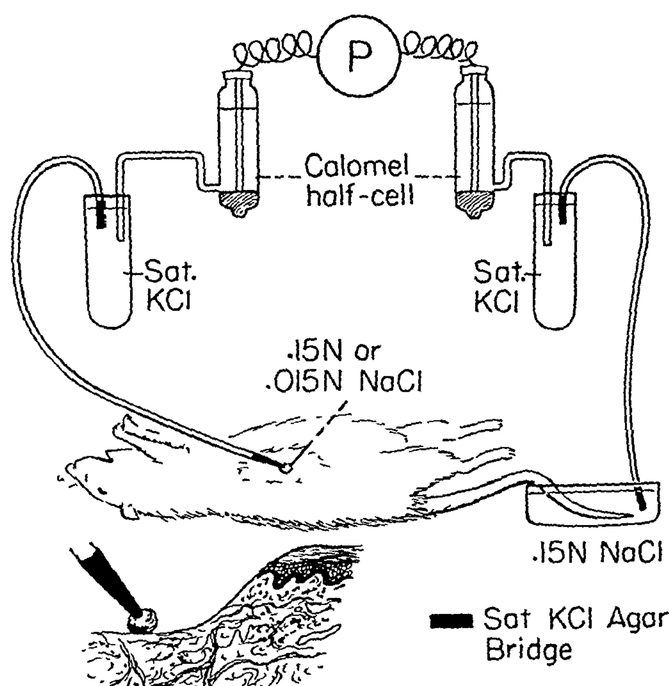


FIGURE 2 Method of studying electrochemically the colloid charge density in rat dermis P is potentiometer connected to saturated KCl-calomel half-cells Rat tail used as reference is immersed in isotonic (0 15 M) NaCl solution. Base-line potential, E_1, is measured with isotonic NaCl buffered at given pH, applied to surface of dermis (*below*) After equilibration, a one-tenth dilution is applied to the dermis, and potential E_2 is determined The difference $(E_2 - E_1)$ depends on the colloid charge density

TABLE 1
DILUTION POTENTIAL AND CHARGE DENSITY OF SOME CONNECTIVE TISSUES

Tissue	E_d (mv)	Density of negatively charged colloid (Eq /kg H_2O)
Cartilage and epiphysis (osteochondral tissue)	+18 to +20	0.160
Skin	0 to −2	0 050
Tendon	−4 to −6	0 035
Synovial fluid	−12	0 010

Significance of titration curves. The titration curve provides certain immediately tangible information about the connective tissue colloids. From it the isoelectric point can be determined and the number of equivalents of dissociable groups above and below this point can be estimated. For any tissue a physicochemical profile, so to speak, is established The effect of changed physiological or pathological states can then be discerned from deviations from

this base line. Moreover, since the shape of the titration curve can be altered by local or parenteral applications of a large number of ions and molecules, the chemical binding of these reagents also can be inferred from it.

The close association of fibrous and amorphous components in connective tissues extends beyond morphologic relations. From the shape of the titration curves (FIGURE 3), as well as from the data on the composition of connective tissue, inferences can be made about interactions at the macromolecular level.[27] Internal electrostatic binding in the matrix may occur between carboxylic acid and amino groups and between sulfate and amino groups. The energy of for-

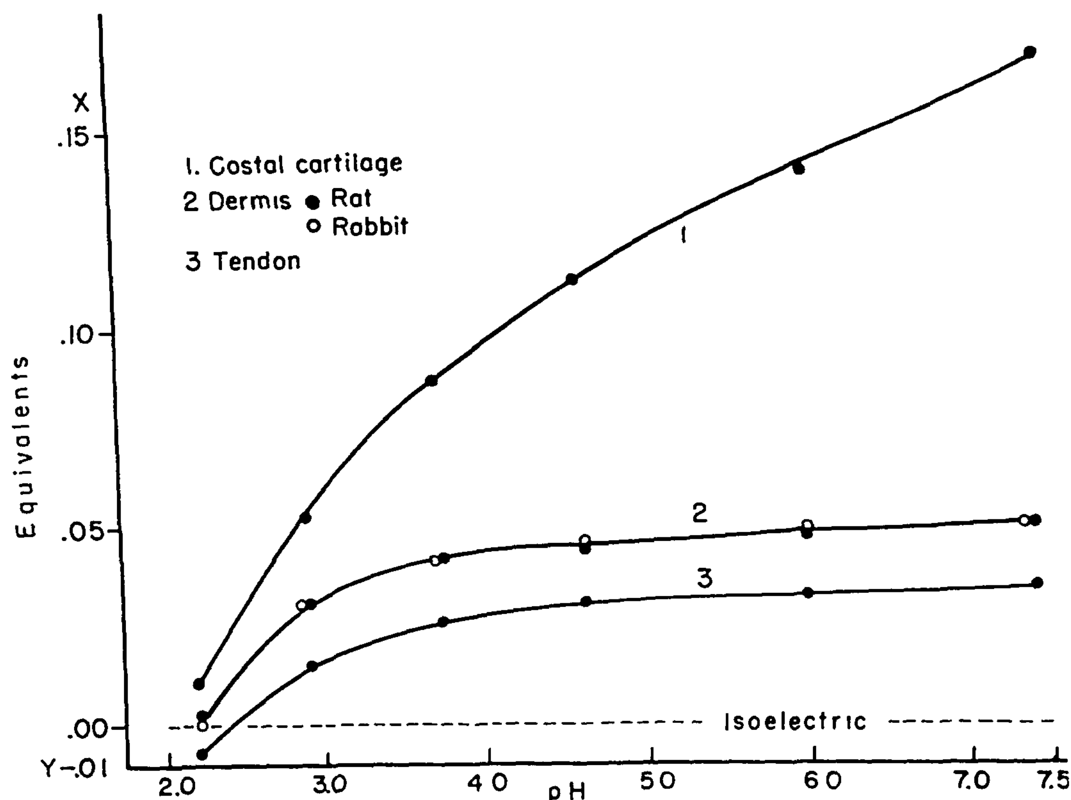


FIGURE 3 Titration curves showing change in charge density with pH for three connective tissues. Ordinate in equivalents per kilogram water X = negative charge, Y = positive charge. Reproduced by permission of *Archives of Biochemistry and Biophysics* [27]

mation of the sulfate-amino bond is evidently higher than that of the carboxylic acid-amino bond. In cartilage where a considerable quantity of chondroitin sulfuric acid is present, the sulfate-amino bond may predominate, and the carboxylic acid groups are free to react with protons; therefore, they are titratable. In skin and tendon (probably also in the gingiva) the internal electrostatic bonds of collagen would predominate. These bonds are essentially between amino and carboxylic acid groups present in approximately equal number. This internal interaction together with the relatively low mucopolysaccharide content would expose only few titratable carboxylic acid groups The configuration of the curves in FIGURE 3 and the changes in the slopes as the hydrogen ion concentration increases form a basis for the foregoing inferences. Also

evident from these curves are the low isoelectric points of connective tissue colloids at *p*H 2.2 to 2.5.

Because of the negative charge on the connective tissue colloids at physiological *p*H, they gain unique properties that affect the distribution of water, electrolytes, and polar molecules. These properties determine further the physiological behavior of tissues.

Donnan equilibrium.[25,27-30] The negative charge is due to an excess of anionic groups, such as the carboxyl and sulfate, on the connective tissue colloid. An immobile net charge is present that must be neutralized by the cations of the *milieu intérieur*, including Na, K, Ca, Mg, and mobile organic cations such as the amines It then follows that these ions will be present at greater concentration in connective tissues than in blood, and this concentration will be determined by the density of the charge, in accordance with the Donnan distribution. For example, with respect to sodium ion, the connective tissue concentration is given by $Na = 0\ 15 + X/2$, where X is the negative charge density. The ratio expressing the relation between tissue concentration and

TABLE 2
APPROXIMATE VALUES FOR PHYSIOLOGICAL CATION CONCENTRATIONS IN BLOOD AND CARTILAGE

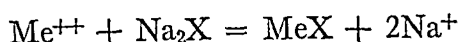
	Blood (mEq /kg. H_2O)	Cartilage (mEq /kg. H_2O)
Negative colloid	10	160 to 170
Sodium	150	280
Potassium	5	70
Ionic calcium	2 5	7.2
Total calcium	5	37
Ionic magnesium	1	3.5
Total magnesium	2	22

blood concentration, (Na tissue/Na blood) = r, is termed the Donnan ratio. In the case of bivalent ions such as calcium, the tissue ion concentration is elevated even further with respect to blood, and (Ca tissue/Ca blood) = r^2. TABLE 2 illustrates these relations by showing the comparative distribution of some cations in cartilage and blood. In the case of potassium, certain special effects are noted that lead to concentrations in connective tissues that are exceptionally high compared to those in blood Here the small hydration shell of the ion is believed to facilitate its interaction with tissue colloids, lowering the activity coefficient of the ion.

With respect to mobile anions such as chloride and phosphate, the Donnan distribution has the effect of lowering their concentration in tissue as compared to that in blood. The ultimate criterion for ion distribution in connective tissues is a thermodynamic one, namely, that the chemical potentials of the various components such as NaCl and KCl in blood and in tissues are the same. This we believe to be synonymous with equilibrium.

Colloid-cation interactions and ion exchange. Many cations interact with connective tissues to form undissociated complexes. With the use of the standard method for measuring the negative charge, this can be demonstrated by the

lowering of the net charge on a tissue surface when these cations are applied. Under the conditions of such an experiment sodium is displaced from the colloid according to the following formulation:



$$Me^{++} + Na_2X = MeX + 2Na^+$$

where Me^{++} denotes a bivalent cation and X denotes the colloid. The physiological bivalent cations, calcium and magnesium, react in this way. Ions such as Sr, Pb, and other polyvalent cations are also bound. Organic cations, including the biological amines, likewise form complexes. Various types of organic anions are also reactive.

Binding of organic anions, cations, and ampholytes involves the same types of electrostatic, hydrogen, and ionic bonds as those occurring among the macromolecular components of the coacervates. There appears to be no essential difference in the bonding between small molecules and ions to the polyelectrolyte structure and that between the various macromolecules. Ionic exchange and histochemical reactions are related to morphology and distribution of

TABLE 3
FREE ENERGIES OF FORMATION OF ION-COLLOID COMPLEXES

Reaction	$\Delta F°$ (cal)
$2K^+ + Na_2X = K_2X + 2Na^+$	−2500
$Ca^{++} + Na_2X = CaX + 2Na^+$	−2300
$Mg^{++} + Na_2X = MgX + 2Na^+$	−2700
$Sr^{++} + Na_2X = SrX + 2Na^+$	−2900
$Pb^{++} + Na_2X = PbX + 2Na^+$	−3500
Histamine + Na_2X = histamine X + $2Na^+$	−3600
Lysine + Na_2X = lysine X + $2Na^+$	−3600
Thiamine + Na_2X = thiamine X + $2Na^+$	−3800

charge of the colloidal matrix. In general, the behavior of the colloids of connective tissue may be likened to that of an exchange resin for ions and molecules.[3,16,25,27-29] The standard free energy, $\Delta F°$, of an exchange reaction may be calculated according to the relations

$$\frac{MeX}{(Me^{++})X^-} = K$$

$$\Delta F° = -RT \ln K$$

where R is the gas constant in calories per degree, T is the absolute temperature, K is the mass-law constant, and ln is the natural logarithm. In TABLE 3 the free energies of exchange of sodium with a number of other cations are given.

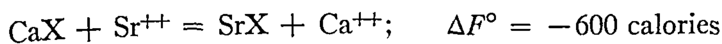
From the foregoing it follows that the cation concentrations in a given connective tissue are dependent upon the negative charge density. The total cation concentration is the sum of the ionic and bound forms. Again the case of cartilage, illustrated in TABLE 2, may be cited. Sodium is present at about twice its concentration in blood, potassium at fourteenfold, calcium at sevenfold, and magnesium at elevenfold. These values, predicted from theoretical considerations, agree closely with the analytic results of Eichelberger *et al.*[38]

The interdependent relations between charge density in the tissue and the total ionic and bound concentrations of an ion in blood and in tissue may be represented best by a series of nomograms such as that for calcium shown in FIGURE 4. To the right is the blood calcium concentration and, to the left, colloidal charge densities in a series of tissues. A line connecting tissue charge densities with normal blood calcium levels intersects tissue values of such other variables as bound calcium, ionic calcium, total calcium, and sodium. Nomograms have been developed for other physiological electrolytes, including magnesium and potassium.[25,28,29] These nomograms, similar in some respects to

FIGURE 4 Nomogram of sodium-calcium equilibrium between blood and connective tissues. Serum sodium is assumed constant at 0 150 Eq /l Varying states of connective tissue, ranging from highly relaxed guinea pig pubic symphysis to normal symphysis or an epiphysis (osteochondral tissue) are denoted

those used by Henderson[39] for his description of blood, emphasize the interdependent relationships among the components of the internal environment of the organism. The various ions must be considered as interacting competitively with connective tissue. Their concentrations are dependent upon the free energy of exchange with sodium and with each other.

It may be seen that the binding of strontium, a cation of radiobiological interest, is stronger than that of calcium:



$$CaX + Sr^{++} = SrX + Ca^{++}; \qquad \Delta F^\circ = -600 \text{ calories}$$

Lead exerts its toxic effects by displacing all the physiological cations (for example, $CaX + Pb^{++} = PbX + Ca^{++}, \Delta F^\circ = -1200$ calories).

Calcification. The foregoing theoretical parts dealing with the Donnan dis-

tribution of ions and with ion binding and ion exchange have important implications for the explanation of calcification in connective tissue. Neither the collagen nor the glycoprotein components should be considered apart as directing calcification. Rather, the properties of the colloidal coacervate, especially the charge density, appear to be of ultimate importance. Through the operation of the Donnan equilibrium calcium ion concentration is related to the square of the immobile charge. Bound calcium is a function of the cube of the negative charge density. It was shown earlier that, in tissues with a high charge density, the total calcium level may rise to values of about seven times the blood level. Shifts in the organization of connective tissue and its charge density are capable of varying locally the amount of bound and ionic calcium. Such shifts, when coupled with changes in phosphate concentrations and in the concentrations of other anions as citrate and bicarbonate, occurring in cellular metabolism, could provide the necessary environment for apatite formation.[28]

Reactions with organic anions and polar molecules. Electrochemical studies provide evidence that connective tissues also interact with organic anions and with uncharged organic molecules that are polar. Reactions of this type already have been well described for the fibrous proteins, wool[40] and collagen,[41] especially in connection with technological problems. The evidence for this is found in the modification in the charge–*p*H curve (titration curve).[27] Following the local application or parenteral administration of various aromatic compounds, for example, salicylate and its congeners, picric acid, and various dyes, the charge on the tissue surface is altered. The reaction may involve such processes as hydrogen bond formation, esterification, and chelation. Moreover, the titration curve, as previously described, is shifted. These studies have led to the concept that the tissue surface is formed of a complex mosaic of negatively and positively charged groups, in addition to a variety of uncharged polar groups that are reactive (FIGURES 5 and 6). Such groups are oriented in some sort of special steric configuration that imposes a conjugate orientation on chemical substances that approach the surface. When a "foreign" molecule such as that of a drug reacts, there is a redistribution of charge affecting the entire field of the surface. In this way the properties of the surface, including its charge and the internal electrolyte distribution in tissues, may be sharply altered by chemical agents and drugs.

FIGURE 6 shows the distribution of the hypothetical ionic atmosphere at the surface in relation to the magnitude of the distances involved. It will be noted that the depth of the double layer is considered to be of the order of 10 Å, which is just beyond the power of resolution of today's electron microscope.

The two-phase system and equilibrium. From studies of the interaction of ions and ground substance it was apparent that the connective tissues were in equilibrium with the blood plasma. When the negative charge density of the ground substance colloids was determined, the distribution of such ions as Na, K, Mg, and Ca between blood and connective tissue could be described adequately by the Donnan equilibrium and the free energy relationships, as mentioned above.

This finding gave rise to a difficulty that had been appreciated by earlier physiologists. Some connective tissues are relatively dense and low in water, others are relatively hydrated and loose, yet both remain in effective equilibrium

Charge distribution on tissue surface

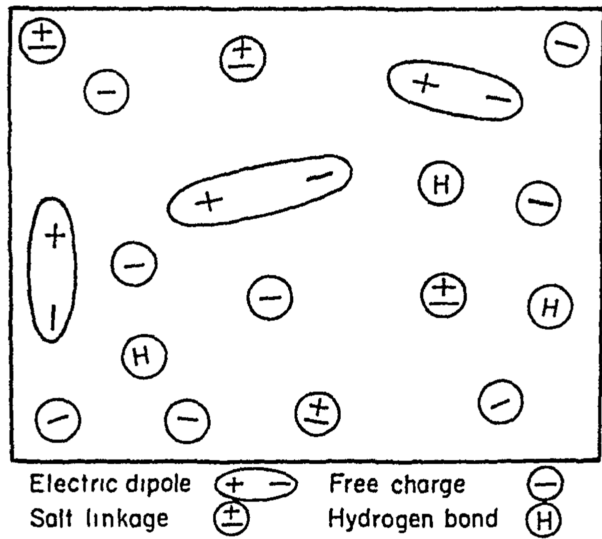


FIGURE 5. Diagram of the charge distribution on a connective tissue surface.

INTERACTIONS AT COLLOIDAL SURFACES

SURFACE

TISSUE

AMBIENT FLUID (μ = 0.15)

0 — 3Å — 6Å — 9Å

← THICKNESS OF ION ATMOSPHERE IN Å →

← MOLECULAR DIMENSIONS →

← LYSINE →

← ALANINE →

← ION DIAMETERS →

TISSUE	Short Range Interactions	Intermediate Range Interactions	Long Range Interactions
Two Phase Amphoteric Colloidal Matrix Proteins Glycoproteins Mucopolysaccharides Aggregated by Internal Bonds Net Negative Charge Dipoles Salt Linkages	Coulombic Forces Salt Linkages Chelation Dipole Interactions Hydrogen Bonds Hydration Esterification Diffusion	Coulombic Forces on Ions and Dipoles	Coulombic Forces on Ions

FIGURE 6. Interactions at connective tissue surfaces and within the ionic atmosphere

with the same blood supply. A single ground substance may contain very different amounts of water at different times, and such changes may occur before synthetic processes have increased the bulk of hydrophilic substances in the tissue. If the ground substance were to be represented by a homogeneous (if complex) solution, then such water differentials should lead to osmotic inequality.

We suggested[16,28] that the ground substance might consist rather of two phases: a colloid-rich, water-poor phase and a water-rich, colloid-poor phase in equilibrium with each other and with the blood plasma (FIGURE 7). This would permit the intervariation of either phase without affecting the equilibrium of the whole. Characteristically, the ground substance may change to the state of increased hydration by the uptake of dialyzate from the plasma and an increase

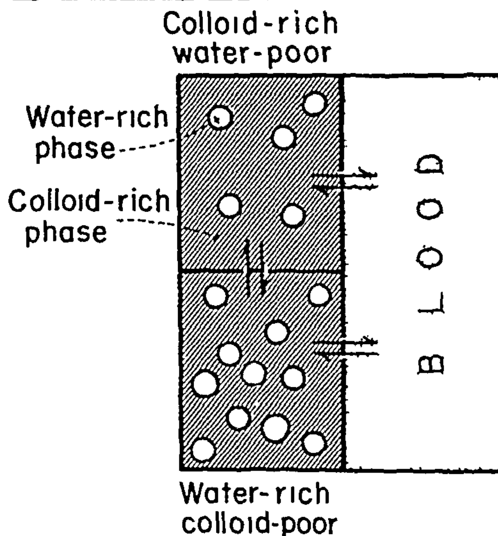


FIGURE 7. Colloidal states of equilibrium within the two-phase system and between blood and the connective tissue

in the water-rich phase at the expense of the colloid-rich phase. The system as a whole continues to be in equilibrium with the blood plasma: that is, vapor pressure and osmotic pressure remain unchanged. There may be, on the other hand, a more or less profound change in the physical character of the ground substance, which changes in bulk and in plasticity.

The Milieu Interne and the Extracellular Compartment

The ground substance is in close proximity to, or encloses, virtually all parenchymatous cells, and it invests and bathes all fibers Since the connective tissue matrix is interposed between blood and cells it represents the route of diffusion of anabolites and catabolites such as water, salts, gases, nutrients, and internal secretions. These diffusible substances are therefore a part of the ground substance at any instant. Their exchanges are largely determined by physical and chemical properties of the extracellular matrix.

How is the classic "tissue fluid" related to the ground substance? There

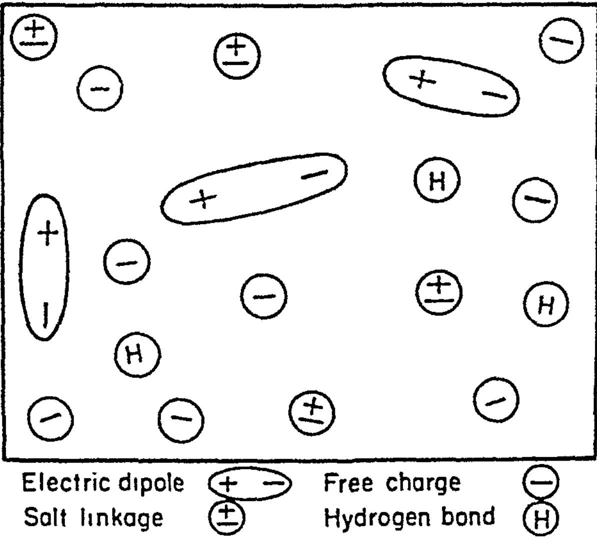


FIGURE 5. Diagram of the charge distribution on a connective tissue surface.

INTERACTIONS AT COLLOIDAL SURFACES

SURFACE

TISSUE

AMBIENT FLUID (μ = 0.15)

0 — 3Å — 6Å — 9Å

THICKNESS OF ION ATMOSPHERE IN Å

MOLECULAR DIMENSIONS

LYSINE

ALANINE

ION DIAMETERS

	Short Range Interactions	Intermediate Range Interactions	Long Range Interactions
Two Phase Amphoteric Colloidal Matrix	Coulombic Forces Salt Linkages Chelation	Coulombic Forces on Ions and Dipoles	Coulombic Forces on Ions
Proteins Glycoproteins Mucopolysaccharides Aggregated by Internal Bonds	Dipole Interactions Hydrogen Bonds Hydration		
Net Negative Charge	Esterification Diffusion		
Dipoles			
Salt Linkages			

FIGURE 6 Interactions at connective tissue surfaces and within the

with the same blood supply. A single ground substance may contain very different amounts of water at different times, and such changes may occur before synthetic processes have increased the bulk of hydrophilic substances in the tissue. If the ground substance were to be represented by a homogeneous (if complex) solution, then such water differentials should lead to osmotic inequality.

We suggested[16,28] that the ground substance might consist rather of two phases: a colloid-rich, water-poor phase and a water-rich, colloid-poor phase in equilibrium with each other and with the blood plasma (FIGURE 7). This would permit the intervariation of either phase without affecting the equilibrium of the whole. Characteristically, the ground substance may change to the state of increased hydration by the uptake of dialyzate from the plasma and an increase

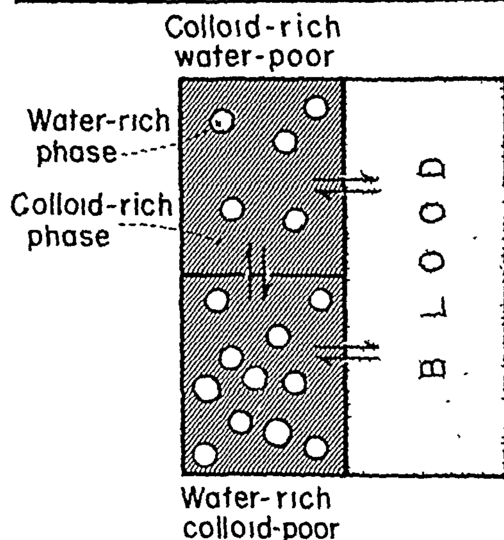


FIGURE 7. Colloidal states of equilibrium within the two-phase system and between blood and the connective tissue

in the water-rich phase at the expense of the colloid-rich phase. The system as a whole continues to be in equilibrium with the blood plasma: that is, vapor pressure and osmotic pressure remain unchanged. There may be, on the other hand, a more or less profound change in the physical character of the ground substance, which changes in bulk and in plasticity.

The Milieu Interne and the Extracellular Compartment

The ground substance is in close proximity to, or encloses, virtually all parenchymatous cells, and it invests and bathes all fibers. Since the connective tissue matrix is interposed between blood and cells it represents the route of diffusion of anabolites and catabolites such as water, salts, gases, nutrients, and internal secretions These diffusible substances are therefore a part of the ground substance at any instant. Their exchanges are largely determined by physical and chemical properties of the extracellular matrix.

How is the classic "tissue fluid" related to the ground substance? There

have been numerous suggested answers, a current one being that the watery portion of the extracellular compartment, the vascular dialyzate, is in some way separate and distinct from the so-called amorphous ground substance with its protein and mucopolysaccharide content In our view, the tissue fluids are coextensive with the ground substance and form an integral part of both its colloid-rich and water-rich phases. In this sense, the ground substance as a whole constitutes the *milieu interne* of cells, forming the extracellular compartment.

It will have become apparent that the ground substance and the blood do not vary independently. Fluctuations in the concentration of any ion in the blood lead to a change in concentration of that ion in the ground substance. Also, physiological or pathological modification of the ground substance, leading to a change in the charge density or character of the colloid, leads to changes in the blood ions. These relationships are adequately expressed by a series of nomograms, as previously explained.

Cells are related to the ground substance directly and to the blood proximately. In this way, the ground substance confers an additional factor of homeostasis to the cell environment. However, changes in the blood and in the ground substance will affect the distribution of substances in relation to the cells, and may also modify the physical environment of cells. The ground substance also is itself a product of cell synthesis, and its amount and nature may be modified by cellular activity. In this way specific interactions of cells and ground substance may be visualized.

Biological Plasticity of Connective Tissue; the Action of Hormones

Connective tissues exhibit great lability in their biological behavior. This may be inferred from the changes in mechanical or viscous-elastic properties that are observed in growth, tissue injury, or pathological change and, especially, in response to hormone stimulation.[1,2,14,16,18,25,28,30,42]

In the following section certain features of this plasticity of connective tissues are discussed, including the general responses to hormones and to variations in physiological and pathological states. This is followed by a description of reactive states in some tissues, with special emphasis on the periodontal tissues.

In the action of hormones on connective tissues two overlapping categories may be denoted. There are hormones in which the targets appear to be chiefly the connective tissues (or their cells) proper Such are the growth hormone, which stimulates the growth of the epiphysial cartilage; relaxin, which promotes the growth and partial dissolution of the symphysis pubis; and cortisone, which affects some general connective tissue target. Other hormones have as their "obvious" targets certain epithelial cells or tissues on which major endocrinological interest has been focused, nevertheless, closer analysis reveals in all cases an action on contiguous or other connective tissues. Thus estrogens not only stimulate the genital tract epithelia in the female but also affect the ground substance of skin, sometimes in a most striking fashion, as seen in the sex skin changes of primates, androgens similarly find their conventional target in the cells of the male accessories, but specifically cause the hypertrophy of the mesenchymal comb and wattles of birds, gonadotropic hormone stimulates the

growth of the ovarian granulosa and simultaneously affects the properties of the ovarian connective tissue stroma. A further fact emerges: estrogen, for example, not only stimulates the connective tissue of the genital tract, its chief target, but also connective tissue remote from this; that is, the connective tissue effect does not depend on the stimulation of contiguous epithelial elements. In the case of some hormones the site of action is in dispute: for example, the parathyroid is held by some to be active at the level of the kidney tubule, by others, on the bone matrix. These points of view are by no means irreconcilable if a more general effect of Parathormone on connective tissue be admitted.

In all the above listings rather ill-defined effects of hormones on "metabolism" must be conceded, but of the loci of such actions very little is known. Ultimately, of course, these changes are ascribed to cellular activity. Connective tissue cells—the fibroblasts, osteoblasts, and odontoblasts—contain in their cytoplasm the precursor materials that form the fibrous and extrafibrillar components of connective tissue in response to hormone stimulation.[1-2,11-12,25,42]

Certain unifying concepts are advanced to describe and to explain the labile behavior of connective tissues as it occurs in response to hormones or in other physiological and pathological processes. In general, we believe that the dominant effect is an increase in the water-rich phase and a corresponding decrease in the colloid-rich phase. This simple concept appears to explain the experimental findings.

The findings may be enumerated as follows: (1) there is usually a change in viscous-elastic properties, such as softening and diminished viscosity; (2) the negative charge density of the tissue colloid, electrometrically determined, is lowered; (3) chemical evidence of a change in the state of aggregation is given by the increased amount of water-soluble carbohydrate-protein complex(es); (4) water and electrolyte distribution are altered; (5) staining with the paS reagent is modified, and basophilia and metachromasia are altered; (6) there is a selective uptake of vitally injected Evans blue dye; and (7) the uptake of intravitally injected ferrocyanide is increased.

With very large (nonphysiological) doses of hormones, the bounds of homeostasis imposed by the two-phase system may be exceeded. This also may be a part of the situation in disease. There may be an accumulation of fluid outside the limits of homeostatic exchange, or a loss or gain of the denser phase. The composition of the blood and of the urine may then reflect these changes.

Gingiva in pregnancy and puberty. A specific example of hormonal influence on periodontal tissues is the gingival change in pregnancy. During this period there is heightened secretion of many hormones, including gonadotropins and steroid hormones. Gingival changes accompany the other somatic alterations but often they are not readily evident. When the connective tissues of the gingiva are studied by the electrochemical method the changes are revealed.[43] During pregnancy the colloidal charge density is about one third lower than the normal level, and it is not restored until some months after parturition. Where there are also such local factors as poor hygiene or poor restorations, the subclinical changes become apparent as "pregnancy gingivitis." Upon histochemical examination with the paS reagents the characteristic changes in the ground substance can be observed: lessening in stainability of ground sub-

stance, attenuation of subepithelial and perivascular basement membranes, and increased amounts of water-soluble glycoproteins (FIGURE 1). Estrogen treatment in primates may cause a simulation of some of these changes.[25]

Gingival swelling also is frequently an accompaniment of the parapuberal period. Here, too, the principal change is an increase in the water-rich phase of connective tissue. The histochemical evidence is virtually identical to that seen in "pregnancy gingivitis," and the condition may be attributed to stimulation from increasing amounts of estrogen or other steroids during this period.

Pathological changes in the gingiva. In pathological states the changes in the ground substance of the gingiva may become intensified, but they follow essentially the pattern already described. Inflammatory reactions are common in the gingiva, because of its exposure to local injury and to the oral bacterial flora. Bacterial enzymes such as collagenases, sulfatases, proteinases, and hyaluronidases can exert their influences on suitable substrates in the fibers and ground substance of the gingiva and lead to dissolution of the structure. Aside from the simple morphologic evidence of such action, there are the usual histochemical findings· a lower density of paS-positive material, thinning of basement membranes, and increased amounts of water-soluble glycoproteins The soluble glycoproteins of inflamed gingival tissues have also been determined by analytic procedures [24] The quantity of soluble glycoprotein present is determined by the method of Winzler *et al.*,[44] and is expressed in terms of its mannose-galactose content Inflamed gingivae show approximately one and one-half times as much soluble glycoprotein as do normal tissues (TABLE 4) In one of the severest forms of gingival disturbance, desquamative gingivitis (or gingivosis), the connective tissue changes are greatly accentuated, sometimes to the point of being irreversible Clinically, the gingivae are red and swollen, there are usually bullae and raw areas; on the slightest manipulation the epithelium peels off from the submucosal layers. From both electrometric studies and histochemical[45] observations it may be concluded that in this condition the connective tissue is profoundly affected. the interepithelial cement is dissolved, the glycoprotein ground substance is disaggregated, and the subepithelial and vascular basement membranes are destroyed The water-soluble mucoprotein fraction is increased, and at the same time there is an uptake of water (edema).

TABLE 4
MEAN VALUES OF WATER-SOLUBLE GLYCOPROTEINS OF DRY DEFATTED GINGIVA EXPRESSED AS CARBOHYDRATE

	Number of cases	Carbohydrate (mg /100 gm)	p*
Normal	15	254 ± 16†	<0 01
Pathological	38	386 ± 25†	<0 01

* Calculated from Fischer's test
† Standard error of mean

Reactive states in bone. The changes that occur in the supporting bone of the teeth and in the contiguous tissues can be understood better in the light of

the general reactions of the ground substance of bone. Ordinarily, undecalcified bone does not give a tinctorial reaction with most stains or with the paS reagents. As a tentative explanation we suggest that the reactive groups of glycoprotein and of collagen are strongly cross-linked and unavailable either for stain binding or for periodic acid oxidation. However, where bone is undergoing resorption, whether induced by local factors or by the action of administrated hormones (as for example, of parathyroid extract) or in the medullary bone of birds during egg-laying, the reactivity with the paS reagent is greatly enhanced, and the resorbing spicules stain a magenta color. This is presumptive evidence of a change in the state of aggregation of the bone matrix.[11-12,14,46] Electrochemical observations verify that the density of negative charge has been lowered. As a corollary, smaller amounts of calcium can be bound. Further confirmation of ground substance disaggregation in bone resorption is given by the higher amounts of water-soluble glycoprotein that can be extracted from the diaphyseal bone in parathyroid-treated animals.[14]

Relations Among Tissue Serum and Urinary Glycoproteins

The question of the origin of elevated levels of serum seromucoid* and of urinary glycoproteins deserves special discussion since it illuminates the labile behavior of connective tissue. Let us consider the ubiquitous distribution of the ground substance in parenchymatous and supporting tissues. Naturally, one would expect that changes in the state of aggregation of connective tissue colloids with the release of soluble materials would be signaled by changes in the blood. In persons with arthritis, rheumatic fever, tuberculosis, or cancer[47-48] the concentration of carbohydrate-containing proteins in the serum is elevated. This might appear to be an utterly nonspecific accompaniment of disease, on the contrary, in all these diseases there is involvement of the connective tissue, either directly or through its role as contiguous stroma.

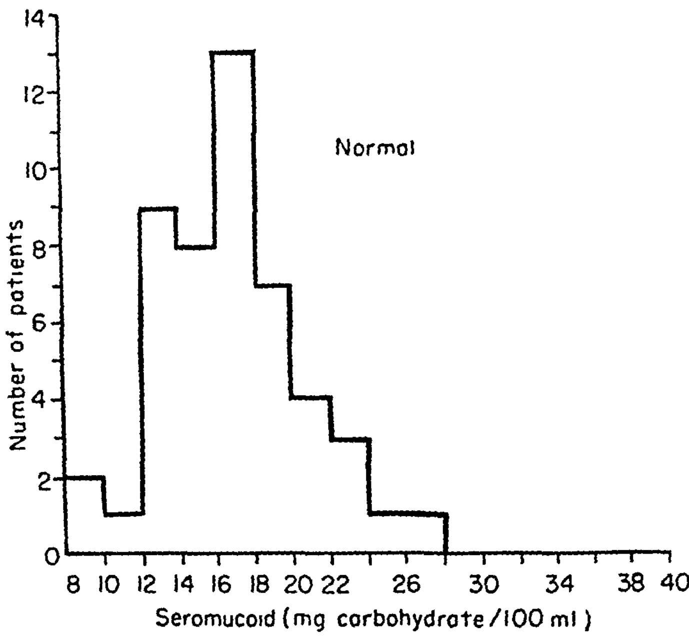
Gersh and Catchpole[1] suggested that the rise in serum carbohydrate-containing proteins represented the diffusion of soluble depolymerized components of connective tissue into the blood. Experimental evidence was obtained in support of this hypothesis. (1) in mice bearing transplanted tumors the stroma contains increased amounts of water-soluble material, and the blood mucoprotein level is elevated,[49] (2) experimental scurvy in guinea pigs is marked by disaggregation of connective tissue ground substance and by an elevated serum mucoprotein level;[50] and (3) following administration of parathyroid extract in animals the mucoprotein ground substance of the metaphysis is disaggregated, the soluble fraction is increased, and the blood seromucoid level rises.[14]

From limited experimental and clinical evidence it appears also that similar urinary changes accompany those in the blood. Thus rats that have received large doses of parathyroid extract excrete in their urine almost three times the normal quantity of mucoprotein.[51] Patients with burns that have caused the destruction and injury of large areas of connective tissue excrete increased

* The group of glycoproteins composing the seromucoid fraction migrates chiefly with the alpha globulins on electrophoretic separation The major fraction is orosomucoid, a glycoprotein characterized by a very low isoelectric point and ready solubility. The hexose concentration of seromucoid, largely mannose and galactose, is about 15 per cent Seromucoid contains no uronic acid

amounts of mucoprotein in their urine.[52] Kidney stone formation in some persons is associated with an increased excretion of the mucoprotein, which has a strong affinity for calcium.

The methods we had used in our earlier studies could not establish the



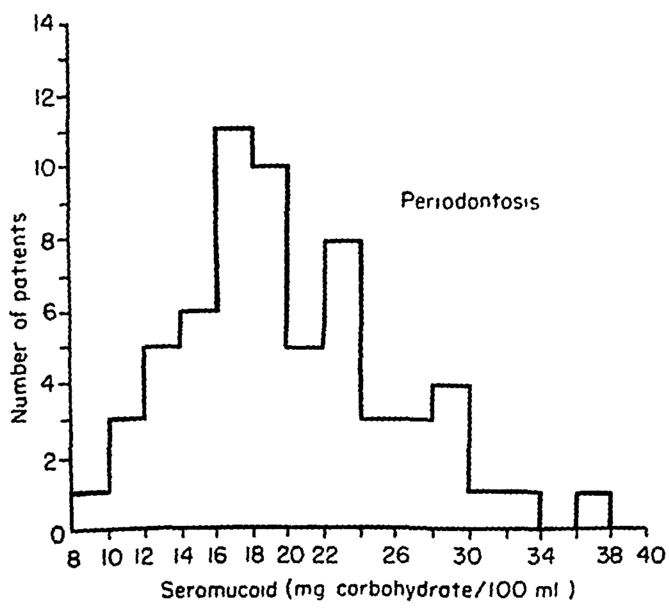


FIGURE 8. Histograms showing distribution of seromucoid levels in normal persons and in a group of patients with periodontosis Reproduced by permission of the *Journal of the American Dental Association* [55]

chemical identity of ground substance carbohydrate-protein complexes with that of the blood. More recently, by the injection of one of the seromucoid fractions into chickens, Silberberg *et al.* succeeded in producing an antiserum to the human blood orosomucoid.[53] This immunochemical method has been adapted to tissue studies and has led to the identification of substances that react with the antiserum to orosomucoid in the soluble fractions of connective tissues from nasal polyps[54] and gingiva. Residual tissue blood had little or no influence on the results. Thus additional evidence of a relationship between certain blood and tissue glycoproteins has been provided.

Seromucoid levels in diffuse alveolar atrophy. The fact that tissue alterations are reflected by blood changes may be of significance in the study of dental disease. In cases of diffuse alveolar atrophy where there is generalized loss of the alveolar bone, it is reasonable to suspect some systemic involvement· perhaps the alveolar bone changes are but the local evidence of a more general disturbance in connective tissue. We have determined serum mucoprotein levels in a group of 62 patients with periodontosis and in another group of comparable age showing no loss of bone.[55] The mean value for seromucoid levels in the serum of the control patients, expressed as mannose-galactose, was 16 8 $\pm$ 3.6 mg./100 ml.; in the group showing diffuse alveolar atrophy it was 19.8 $\pm$ 5 5 mg./100 ml. ($p < 0.001$). Approximately one third of the latter group had elevated seromucoid levels of 22 mg./100 ml. or higher, that is, values in excess of one and one-half times the standard deviation of the control group (FIGURE 8).

The elevated seromucoid levels in many patients suffering from generalized loss of alveolar bone could be attributed either to the liberation of diffusible glycoproteins caused by the local destruction of bone, periodontal membrane, and cementum or to a more general process embracing the entire connective tissue system. We have not been able to discriminate between these two possibilities, but it appears reasonable to assume that changes in the periodontium alone could not produce the observed blood changes. In our opinion, the oral changes in diffuse alveolar atrophy are more likely to be a manifestation of a general change in connective tissues.

Summary

Combined morphologic, chemical, and physicochemical studies have been made on a variety of connective tissues, including periodontal tissues. The relations between cells, fibers, and the extrafibrillar ground substance were discussed. The connective tissue ground substance is treated as the strategic extracellular substance intermediate between cells and blood; it plays a dominant role in homeostasis. Early theoretical considerations led to the hypothesis that connective tissues are organized as heterogeneous colloids containing at least two phases in equilibrium with each other: a water-rich, colloid-poor phase and a colloid-rich, water-poor phase. The predictions were confirmed by electron microscopy in the laboratory of I. Gersh.[15,17,18]

Connective tissues carry an immobile negative charge at physiological *p*H because of the relatively high concentration of acidic groups, including carbohydrate-containing substances. Changes in this charge with *p*H, as electro-

amounts of mucoprotein in their urine.[52] Kidney stone formation in some persons is associated with an increased excretion of the mucoprotein, which has a strong affinity for calcium.

The methods we had used in our earlier studies could not establish the

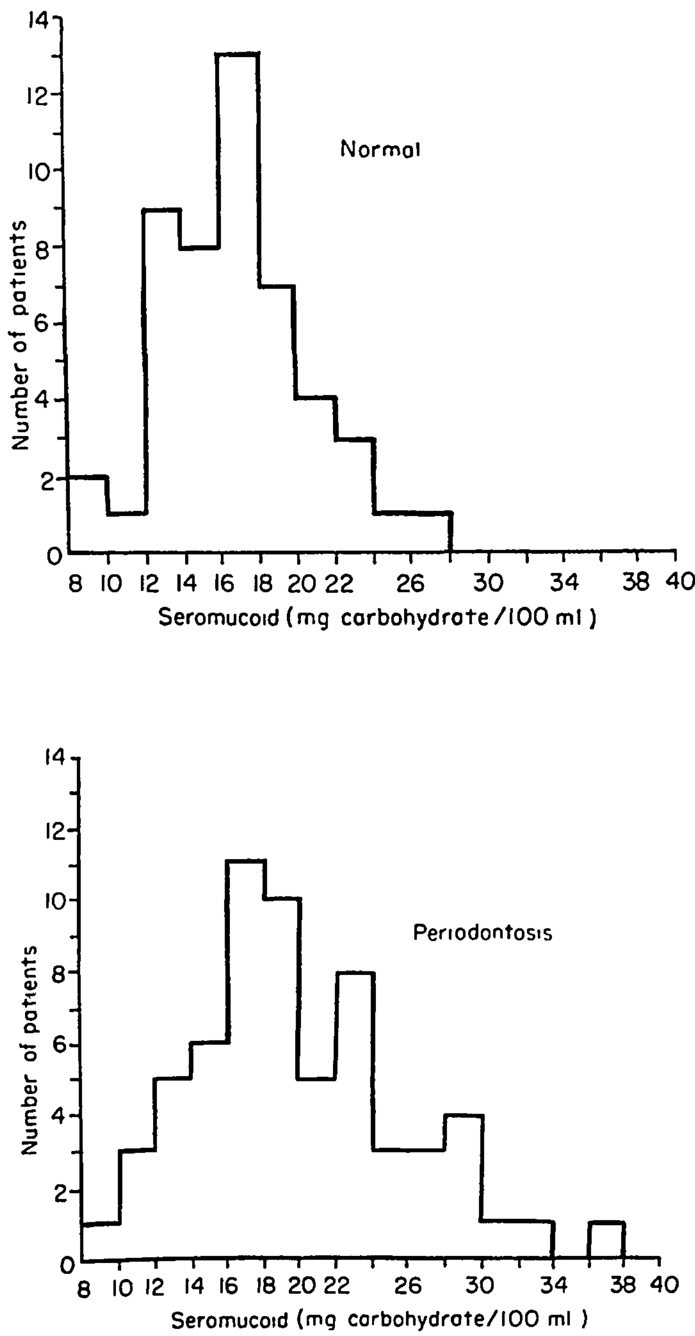


FIGURE 8 Histograms showing distribution of seromucoid levels in normal persons and in a group of patients with periodontosis Reproduced by permission of the *Journal of the American Dental Association* [55]

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metrically determined, constitute an *in vivo* titration curve of a tissue. Tissues have their distinctive titration curves, reflecting in part their chemical components and the nature of the stabilizing internal bonds. Connective tissues have the attributes of ion-exchange resins, and they accumulate cations in relation to their charge densities. Electrolyte equilibrium between blood and connective tissues has been shown to depend upon the density of negative charge, upon the Gibbs-Donnan equilibrium, and upon the free energies of formation of ion-colloid complexes. Polar organic molecules and organic anions also react with the surface of the connective tissue colloids through short-range forces such as hydrogen bonding, esterification, and chelation. Histochemical reactions of connective tissues depend on the colloidal properties.

Labile behavior of connective tissues in response to hormones, growth, or injuries depends in part on the breaking of low-energy bonds, with a concomitant change in the state of aggregation of the colloid. Changes in the periodontal tissues in pregnancy, puberty, gingivitis, desquamative gingivosis, and diffuse alveolar atrophy are discussed in the light of the general theory described.

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RELATIONS BETWEEN THE METABOLISM AND STRUCTURE OF BONE*

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Despite its rigid physical character, bone, like most other connective tissues, is a highly labile substance capable of rapid structural transformations. Although these morphologic changes have been investigated extensively, the means by which they are accomplished still is understood poorly.

There are numerous places in nature, other than in bone, where problems of solubilization also exist.[1-3] In many of these instances, it has been suggested that the dissolution of the insoluble matter occurs through chelation or complex formation with certain of the intermediate products of carbohydrate metabolism.[2, 4-6] We have considered the possibility that a similar situation exists in bone resorption. Thus, it has been postulated that accumulated di- and tricarboxylic acids could compete for cations with the mucoproteins and other negatively charged colloids of the bone matrix, thereby bringing the calcium salts into solution.[7-9] To develop this hypothesis, certain aspects of the carbohydrate metabolism of bone were studied during the metaphyseal dissolution caused by parathyroid extract and in the sequence of apposition and resorption in pigeon bone during egg-laying.

Respiration and Anaerobic Glycolysis of Bone

Since the formation of di- and tricarboxylic acids occurs during the oxidative degradation of carbohydrates, our initial studies were concerned with the effects of parathyroid hormone upon this phase of cell metabolism.[9] For these experiments, the resorptive state was induced in weanling rabbits by the administration of 1000 U. of parathyroid, U.S.P. grade, intramuscularly in divided doses 42 and 28 hours before sacrifice. The respiration of the metaphyseal bone slices from the tibias and femurs of these animals was measured in the Warburg respirometer, using standard manometric techniques. Similar studies also were performed with bone slices from normal rabbits.

The results of these experiments are given in TABLE 1. Since the metabolically inert bone matrix forms the largest part of the tissue mass, it was considered a more reliable index of cellular activity to relate the data to the desoxyribonucleic acid (DNA) content of the tissue rather than to the dry weight. It has been shown that the DNA content per nucleus is the same for most cells.[10] When expressed in this manner, the oxygen consumption of the bone cells from the hormone-treated animals was shown to be reduced approximately 40 per cent. Conceivably, such a decrease in respiration could lead to the accumulation of certain di- and tricarboxylic acids in the cells and extracellular environment.

The rates of endogenous anaerobic glycolysis in bone from normal and Para-

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This difference in dehydrogenase activity also could be demonstrated qualitatively in the gross specimens of long bones from rabbits perfused through the descending aorta with 500 cc. of 0.1 per cent triphenyltetrazolium chloride. In the control animals the entire metaphyseal region was stained deep red, while in the animals having received parathyroid extract (two 500-unit doses over a 44-hour period), only small amounts of the insoluble red formazan were noted.

Since metaphyseal bone slices contain a quantity of marrow, the possible contribution of these cells to the tetrazolium reaction must be considered. Studies performed with whole marrow preparations from normal and Parathormone-treated rabbits, however, revealed the fact that the total amount of TTC reduced in each instance was considerably less than that measured with the bone slices (TABLE 4). Moreover, no significant differences existed between the two groups. These findings indicated that the hematopoietic elements contributed very little to the total reduction of TTC.

TABLE 3

REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY METAPHYSEAL BONE SLICES FROM NORMAL AND PARATHYROID-TREATED RABBITS

	No of experiments	Substrate*	Amount TTC reduced†	
Control	10	Succinate	75 3 ± 6.8 μg.	$p < 0.01$
Treated	10	Succinate	30.8 ± 3 6 μg.	

* Sodium succinate substrate in final concentration of 0 033 *M*.
† Expressed as micrograms of triphenyltetrazolium chloride reduced per 35 min. per milligram of DNA; means and standard deviations.

TABLE 4

REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY BONE MARROW FROM NORMAL AND PARATHYROID-TREATED RABBITS

	No of experiments	Substrate*	Amount TTC reduced†
Control	10	Succinate	23 ± 16 μg.
Treated	9	Succinate	26 ± 16 μg

* Sodium succinate substrate in final concentration of 0 033 *M*.
† Expressed as micrograms of triphenyltetrazolium chloride reduced per 35 min per milligram of DNA; means and standard deviations.

The suppressive effect of parathyroid hormone upon dehydrogenase activity in bone provides a possible explanation for the reduction in the rate of aerobic carbohydrate degradation. Since sodium succinate was used as the substrate in these experiments, it was presumed that succinic dehydrogenase activity was affected predominantly. On this basis, any or all of the di- and tricarboxylic acid intermediates preceding fumarate in the Krebs cycle could accumulate at sites of bone resorption.

thormone-treated rabbits were similarly investigated.[9] In contrast to the previous findings, no significant difference was observed between the two groups (TABLE 2). This observation indicated that the depression of respiration was a specific effect rather than merely the result of a toxic reaction of the bone cells to the large doses of parathyroid extract.

Histochemical studies of representative sections of metaphyseal bone treated by the periodic acid-leukofuchsin method revealed alterations in staining properties that in all probability could be related to the changes observed in aerobic carbohydrate metabolism.[9] Thus, while in normal bone the contiguous connective tissue cells and many of the osteoblasts and osteocytes contained aggregates of glycogen, in the bone from the parathyroid-treated rabbits the amount of intracellular glycogen was reduced significantly. Since the

TABLE 1

ENDOGENOUS RESPIRATION OF METAPHYSEAL BONE FROM NORMAL AND PARATHYROID-TREATED WEANLING RABBITS

	No. of experiments	Mean Q_{O_2} (DNA)* (± S.D.)	
Control	67	−57 ± 4	$p < 0.01$
Treated	32	−33 ± 11	

* Expressed as microliters of oxygen utilized per hour per milligram DNA

TABLE 2

ANAEROBIC GLYCOLYSIS OF METAPHYSEAL BONE FROM NORMAL AND PARATHYROID-TREATED WEANLING RABBITS

	No. of experiments	Mean $Q_G^{N_2}$ (DNA)* (±S.D.)	
Control	18	+33.2 ± 15.4	$p < 0.05$
Treated	19	+27.8 ± 9.8	

* Expressed as microliters of CO_2 formed per hour per milligram DNA.

oxidative degradation of carbohydrates was limited by parathyroid hormone, anaerobic glycolysis, which is a less efficient means of obtaining energy, would be expected quickly to deplete the glycogen stores.

Dehydrogenase Activity of Metaphyseal Bone

In order to gain information about the possible site of action of parathyroid hormone upon oxidative carbohydrate metabolism, dehydrogenase activity also was studied in metaphyseal bone from treated and untreated rabbits.[9] The tissue slices were incubated with triphenyltetrazolium chloride (TTC), using a sodium succinate substrate, and the amount of reduced TTC was measured spectrophotometrically by a modification of the method described by Kun and Abood.[11] The results showed that the metaphyseal bone slices from the hormone-treated animals reduced approximately 60 per cent less TTC than did the normal slices (TABLE 3).

This difference in dehydrogenase activity also could be demonstrated qualitatively in the gross specimens of long bones from rabbits perfused through the descending aorta with 500 cc. of 0.1 per cent triphenyltetrazolium chloride. In the control animals the entire metaphyseal region was stained deep red, while in the animals having received parathyroid extract (two 500-unit doses over a 44-hour period), only small amounts of the insoluble red formazan were noted.

Since metaphyseal bone slices contain a quantity of marrow, the possible contribution of these cells to the tetrazolium reaction must be considered. Studies performed with whole marrow preparations from normal and Parathormone-treated rabbits, however, revealed the fact that the total amount of TTC reduced in each instance was considerably less than that measured with the bone slices (TABLE 4). Moreover, no significant differences existed between the two groups. These findings indicated that the hematopoietic elements contributed very little to the total reduction of TTC.

TABLE 3

REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY METAPHYSEAL BONE SLICES FROM NORMAL AND PARATHYROID-TREATED RABBITS

	No of experiments	Substrate*	Amount TTC reduced†	
Control	10	Succinate	75.3 ± 6.8 μg.	$p < 0.01$
Treated	10	Succinate	30.8 ± 3 6 μg.	

* Sodium succinate substrate in final concentration of 0 033 *M*.
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roid hormone-treated animals has a greater water content and cell-matrix ratio than normal, a meaningful comparison cannot be made on this basis. The data, therefore, were expressed also in terms of the DNA content of the tissue. Related in this manner, the citric acid extracted from normal bone slices gave a mean value of 862 cpm/mg. DNA, compared with 2015 cpm/mg. DNA for the samples from the parathyroid-treated rabbits. These results definitely established the fact that an increased concentration of metabolically formed citrate is present at sites where parathyroid-induced resorption is occurring.

Serum Citrate Concentrations in Apposition and Resorption

Since bone resorption produced by exogenous parathyroid extract is a relatively nonphysiological state, several studies involving citrate metabolism also were performed, using the egg-laying pigeon. Bloom *et al.* were among the first to recognize the value of using this bird as a means for studying grossly exaggerated physiological transformations in bone.[26] Here, within a matter of days, marked apposition and resorption succeed each other in rapid succession. For about 1 week preceding the laying of the first egg (a clutch of two eggs is laid in approximately a 40-hour period), the hollow medullary cavities of the tibia and femur become filled with bone. A day before the egg is laid, while the shell is being calcified, much of this bone is resorbed and the marrow cavities are partially restored. In the subsequent 40-hour period associated with calcification of the shell of the second egg, the phases of apposition and resorption are repeated. Finally, the bird goes into a resting phase that lasts until the beginning of the next ovulatory cycle. During active periods this phase may take only 3 to 7 days.

TABLE 6

SERUM CITRATE LEVELS DURING THE PIGEON EGG-LAYING CYCLE

No of experiments	Phase	Citrate concentration	p
24	Resting	5 8 ± 2 9 mg /100 ml	
29	Apposition	6 6 ± 1 4 mg./100 ml	< 0 001
39	Resorption	8 7 ± 1 3 mg /100 ml.	

In our initial experiments blood citrate concentrations were measured during these cyclic medullary bone changes. The birds were mated and their shell glands palpated daily until an egg was felt. With this as a point of reference, blood samples were drawn from the wing vein at intervals corresponding to the various phases of the egg-laying cycle. Citrate determinations were made on 0.2-ml. aliquots of serum using L'Heureux and Roth's modification of the method of Natelson *et al.*[20]

In the resting phase the average serum citrate level was 5.8 mg./100 ml., while in the appositional phase the level increased slightly to a mean of 6.6 mg./100 ml. (TABLE 6). During resorption accompanying shell formation, the citrate concentration rose to its highest level, averaging 8.7 mg./100 ml. Thus it was evident that, in the pigeon, blood citrate levels rise while bone is being

Citrate Formation in Metaphyseal Bone

Of the possible tricarboxylic acids to be considered, citrate has received the most attention. There are several reasons for this emphasis. First, the low dissociation constant of calcium citrate favors the solution of insoluble calcium salts in the presence of this anion.[12-14] Second, experiments on the equilibration of apatite systems and powdered bone with buffered citrate have shown that soluble complexes can be formed with bone mineral.[15-18] Moreover, histological changes resembling those that occur with Parathormone have been demonstrated after the intravenous administration of neutral sodium citrate,[19] Finally, an elevation of the serum citrate level has been reported following injection of parathyroid extract,[17, 20] and also in hyperparathyroidism.[21] Conversely, it has been shown that the serum citrate concentration decreases after parathyroidectomy.[7, 22]

To determine whether the diminished respiration that was measured in bone from Parathormone-treated animals results in the accumulation of citrate. Lekan, in our laboratory, studied the conversion of C^{14}-labeled sodium pyruvate to citrate in metaphyseal bone slices from normal rabbits and from a group in which resorption was produced by injection of 1000 U. of parathyroid, U.S.P. grade, in divided doses over a 48-hour period.[23, 24] Utilizing this method, a distinction could be made between citrate formed through cell metabolism and that merely liberated from the apatite phase during the resorptive process.

Table 5

C^{14}-Labeled Citrate Formed from Pyruvate-2-C^{14} by Bone Slices from Normal and Parathyroid-Treated Weanling Rabbits

	No of experiments	cpm/gm bone*	cpm/mg DNA†	
Control	4	3390	862	$p < 0.05$
Treated	4	10700	2015	

* Mean counts per minute per gram of wet bone
† Mean counts per minute per milligram of DNA

Approximately 1500 mg. of bone from the tibia and femur were incubated for 4 hours in a Krebs-Ringer bicarbonate buffer, *p*H 7.4, with 100 λ (1×10^6 counts per minute) of sodium pyruvate-2-C^{14} at 37 5° C. The reaction was stopped by adding perchloric acid, and the radioactive CO_2 was absorbed with KOH The tissue slices were homogenized and washed several times with additional perchloric acid. The DNA content of the residue was determined The combined supernatants were neutralized with KOH and passed through a Dowex-1 (formate form) anion exchange column. Citric acid was eluted with formic acid according to the method of Busch *et al.*[25] The presence of citrate in the eluate was verified quantitatively by a colorimetric method.[20] The radioactivity of the plated citrate fractions was measured in a free-flow gas counter.

When based upon the wet weight of the slices, the mean value in counts per minute (cpm) per gram of bone was 3390 for the normal animals and 10,700 for the experimental group (Table 5). However, since bone from parathy-

account stoichiometrically for the total structural change that occurs in bone resorption, such changes can be envisioned readily when it is considered that the measurements represent merely one phase in a dynamic process.

Discussion

In the past, studies of carbohydrate metabolism in bone have dealt primarily with the enzymes involved in anaerobic glycolysis and their importance in the calcification process. With the increased emphasis upon the relation between citrate and bone resorption, however, considerable attention has been given recently to the aerobic phase of carbohydrate degradation As a result of such investigations it now has been established that bone contains the enzyme systems necessary for the function of the Krebs cycle.[9,30,31] The presence of this metabolic pathway in bone is substantiated by the recent report on the identification of the various di- and tricarboxylic acid intermediates after incubation of tissue slices with C^{14}-labeled acetate.[32]

The ability of parathyroid hormone to suppress aerobic carbohydrate metabolism in bone has been demonstrated in our experiments[9] Although it is still not clear whether this occurs by destruction of the chromophoric group of coenzyme II, as claimed by Neuman *et al.*,[33] by inhibition of succinic dehydrogenase activity, or by a more general effect on intracellular proteins, the end result in any instance could be the same, namely an accumulation of citrate in the extracellular environment. Such an increase in metabolically formed citrate was shown in our studies on the conversion of C^{14}-labeled pyruvate.[23,24]

In order to consider the possible effects of citrate accumulation upon bone, the interrelations between its various components must be examined first. Bone may be characterized as a complex aggregate consisting of colloids, apatite, electrolytes, and water. Under physiological conditions, the mucopolysaccharides, glycoproteins, collagen, and other constituents of the colloidal phase form a coacervate that imparts to bone a high density of relatively immobile negative charge As a result of this negative charge, bone matrix is able to bind greater quantities of calcium than other connective tissues.[8] This protein-bound calcium is in equilibrium with the crystalline (apatite) and ionic phases The structure of bone depends, not only upon the relative proportions of these three fractions, but also upon the interactions between them and the other components.

Any change in the composition of the surrounding body fluids or the metabolism of the contiguous cells can influence the structural integrity of bone. Thus, the accumulation of a metabolite such as citrate, which has the ability to form soluble compounds with calcium, can lead readily to the characteristic changes of bone resorption by removing this cation from the ionic, crystalline, and protein-bound fractions. Not only will the mineral phase be affected in this manner, but the removal of calcium from its combination with protein will result also in a disaggregation of the organic matrix (FIGURE 1). Changes in the solubility of the protein also may be achieved by a direct interaction with citrate. This has been observed in the *in vitro* studies on the solution of collagen[34]

Since bone contains a large quantity of citrate as part of the apatite phase,

resorbed and are lower during appositional and resting phases. While most of this citrate probably originates from dissolved bone matrix (bone contains more than 1 per cent of the dry weight as citrate), it is evident that some also reflects the previously noted changes in cellular carbohydrate metabolism.

Soluble Citrate in Pigeon Bone During Egg-Laying

The concentrations of citrate in the metaphyseal bone during the various phases of the egg-laying cycle were also investigated.[27] Previous attempts to demonstrate changes in the amount of metabolically formed citric acid during periods of active bone resorption have been complicated by the use of whole bone preparations.[28, 29] It is evident that with such experiments, possible variations in intracellular citrate could be masked by the large amounts of insoluble citrate liberated from the bone matrix. To overcome this difficulty, an aspiration procedure was used that largely sequestered cells and soluble extracellular material.[27]

For these experiments, mated pigeons were sacrificed during the resting, appositional, and resorption phases. The exact period was confirmed by gross

TABLE 7
SOLUBLE CITRATE ASPIRATED FROM PIGEON MEDULLARY BONE

No of birds	Stage	Citric acid* (μg /gm)	p
8	Resting	167 ± 82	
7	Apposition	846 ± 203	< 0 001
15	Resorption	1361 ± 304	

* Mean values and standard deviations expressed as micrograms per gram of lyophilized material

examination of the ovaries and ovulatory tracts and gross and histological examination of the long bones. Material for citrate analysis was obtained from the metaphyseal region of the tibia and femur by aspirating across the cut surface of the bone with a fine-bore glass tube covered with a 100-mesh wire screen. By this procedure, bone particles were excluded. The aspirated matter was homogenized and then lyophilized. Citrate determinations were performed on 50-mg. portions. The results, expressed as micrograms of citric acid per gram of dry aspirated material, represented a measure of intracellular citrate plus any liberated extracellular fraction (TABLE 7).

During the resting phase the citrate concentration was 167 μg./gm. During the appositional phase this level increased about five times (846 μg./gm). In the resorptive stage the highest concentrations occurred (1361 μg./gm.), representing an eightfold increase over the resting level. These data also could be expressed in terms of tissue water, making the assumption that the extracted cellular material had an 80 per cent water content. Then the value was 4.2 mg./100 ml. in the resting stage, 21.2 mg /100 ml. during apposition, and 34.0 mg./100 ml in the resorptive phase Thus, during periods of activity in the bone, the local citrate concentrations exceed the serum levels by 3 to 4 times. Although it would seem that such quantities of citrate might not

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there has also been considerable speculation concerning its possible role in bone formation. Because of the high concentration, it seems unlikely that the citrate present is derived only from the blood. In our studies of soluble citrate concentrations in the bone of egg-laying pigeons, the level was elevated slightly during the appositional phase. We assumed that this rise represented citrate of local origin related to increased carbohydrate metabolism accompanying matrix formation. In support of this view are the findings of Lees and Kuyper,[32] who showed that most of the other Krebs's cycle intermediates are also present in the insoluble fraction of bone in greater amounts than in the blood or in an active tissue such as liver. Since citrate in certain concentrations can form insoluble as well as soluble compounds with calcium phosphate,[15, 16] it is conceivable that during bone formation the presence of this

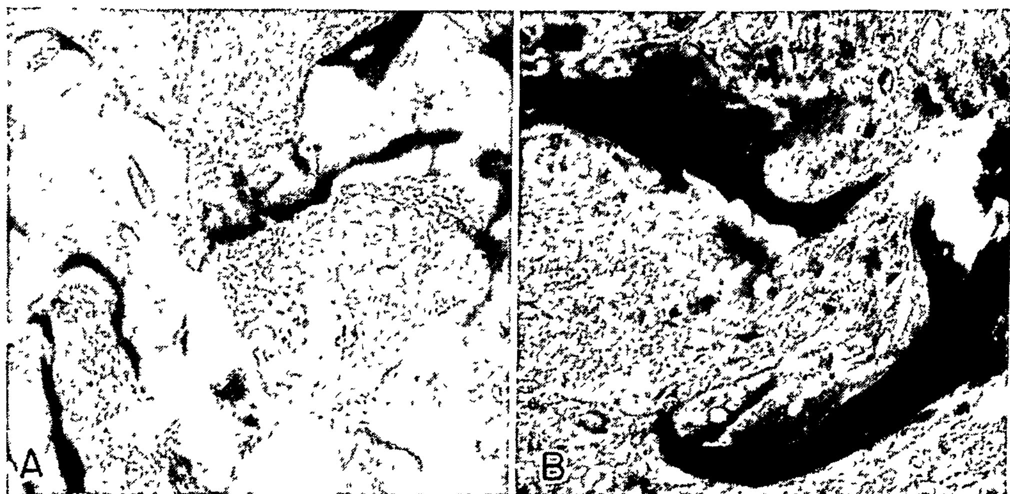


FIGURE 1 Pigeon medullary bone stained by the periodic acid-leukofuchsin method ×560 (a) Appositional phase Only the new bone on the periphery of the spicules is deeply stained This is believed to represent a relatively low state of aggregation and a less highly organized state of the colloidal matrix of bone at sites of new formation (b) Resorptive phase. The entire spicule is stained This is believed to denote a disaggregation of the colloidal matrix throughout the bone, generally characteristic of the resorptive state.

anion could favor apatite formation.[27, 35] Thus, there exists the possibility that citrate may have a regulatory function in both apposition and resorption.

The emphasis in this report has been placed upon citrate However, it is likely that additional anions of metabolic origin also may participate in bone resorption and apposition. For example, the importance of the other di- and tricarboxylic acids found in bone must be considered. Likewise, the contribution of tissue breakdown products such as the uronates and nucleates[36] and the influence of the carbonate ion[37] upon these processes also must be assessed. These problems, however, do not detract from the present hypothesis. On the contrary, they emphasize the idea that basically the structural transformations in bone are governed by alterations in cell metabolism.

Acknowledgment

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HISTOCHEMICAL OBSERVATIONS ON ENZYMATIC PROCESSES IN BONES AND TEETH

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Introduction

Enzymes are considered to be the biological catalysts that control the rate of chemical reactions in bone as well as in other tissues. Definitive study of enzymes in hard tissues by means of routine biochemical procedures presents certain technical problems, since calcifying tissues are nonhomogenous systems containing several different types of cells and intercellular substance. On the other hand, microscopic histochemical techniques appear to offer excellent possibilities for visualizing and studying specific enzymes of these various cellular components.

Until recently alkaline phosphatase was given foremost consideration in histochemical studies. This is due primarily to the impetus given to modern microscopic histochemistry by the original phosphatase technique of Gomori.[1] Application of the aforementioned technique in the study of calcifying tissues has been reviewed in the text edited by Bourne.[2] More recently histochemical localizations of a number of hydrolases associated with the development of hard tissues have been reported.[3]

Fixation Procedures

According to Chessick[4] and Gomori,[5] diffusion of enzymes may be one of the causes of false localizations in unfixed frozen sections. Additional data also support this view.[6] The use of unfixed frozen sections presents problems related not only to the undenatured state of the tissue, but also to the solubilizing effect of freezing and thawing upon enzymes. Whenever feasible, some type of fixation should be employed in the study of hydrolytic enzymes. Cold formalin-fixed frozen sections and cold acetone-fixed paraffin-embedded tissues have been most generally employed in the past.[1] I have employed the freeze-dry technique primarily for the study of hydrolases in bones and teeth.[3] Freezing and drying is usually combined with a plastic-paraffin double-embedding technique. This procedure not only enhances preservation of tissue elements, but enables paraffin sections to be floated on a water bath without disruption of the section or loss of enzyme components.

In the case of oxidases and dehydrogenases, these enzymes are too sensitive to withstand most types of fixation; frozen sections or freshly cut slices of tissue must be employed. Fortunately, enzymes such as cytochrome oxidase are very insoluble, and the use of frozen sections for their demonstration is satisfactory.

The tissues used consisted of heads and knee joints of immature (1 to 6-day-old) rats, mice, and hamsters. Utilizing the freeze-dry double-embedding technique, it is possible to section these specimens without prior decalcification. In most instances frozen sections can also be cut for use with the oxidase and dehydrogenase techniques.

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FIGURE 2 Alkaline phosphatase activity of osteoblasts associated with early stage of intramembranous bone formation in 2-day-old hamster head Noncoupling technique employing 5,6,7,8-tetralolcarbcxylic acid-β-naphthylamide Clear ovoid unstained areas are nuclei ×143

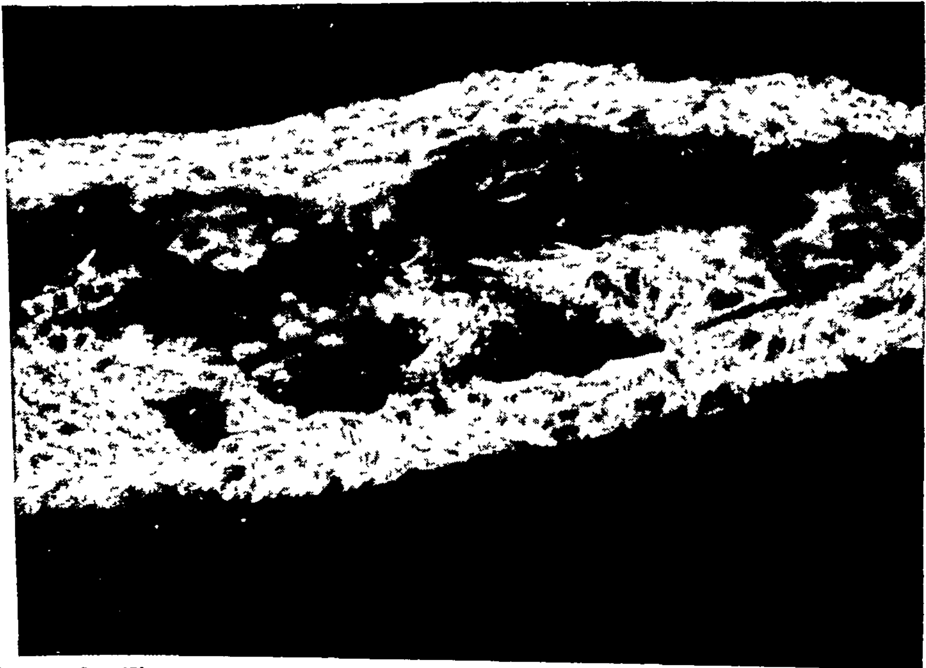


FIGURE 3 Fluorescence technique for alkaline phosphatase, showing activity of osteoblasts associated with palatal bone formation (3-day-old hamster). Same substrate as described in FIGURE 2 ×80.

Specific Enzymes

Alkaline phosphatase, 5-nucleotidase, and adenosine triphosphatase As indicated above, alkaline phosphatase in calcifying tissues has been studied rather intensively from the histochemical standpoint.[2] Most of the previous studies have employed the classic Gomori glycerophosphate technique. Although this procedure is capable of giving good results, Gomori[5] has shown that, in the case of bone, diffusion of the reaction products may give false localizations. In addition, he feels that the nuclear staining that may be present around sites of high activity represents a diffusion artifact. It is also possible that phosphate in bone may be nonspecifically "stained" by the glycerophosphate technique.



FIGURE 1 Alkaline phosphatase activity of endosteum and periosteum of 3-day-old hamster Naphthol AS-MX phosphate Nuclear counterstain is hematoxylin ×143.

This report deals with azo-dye procedures employing complex naphthol adenosine AS-phosphates. Highly insoluble dyes are formed, and the tendency toward diffusion is minimized.[6,7] The AS-naphthols, derived from 2-hydroxy, 3-naphthoic acid and an arylamine, produce some of the most insoluble and chromogenic azo dyes known.[8] With naphthol AS-phosphates it is possible to incubate tissue sections over a *p*H range of 5 to 9 and obtain good localizations. On the other hand, the glycerophosphate technique may exhibit diffusion artifacts at a nonoptimal *p*H (*p*H 7). A large series of simple and complex naphthol AS-phosphates gives identical localizations over a *p*H range of 7 to 9. Similar localizations were obtained using new postcoupling, noncoupling, and fluorescence procedures.[9] In the noncoupling technique the phosphate ester releases upon enzymatic hydrolysis a naphthol that is opaque or semiopaque. With the fluorescence technique the released naphthol, in

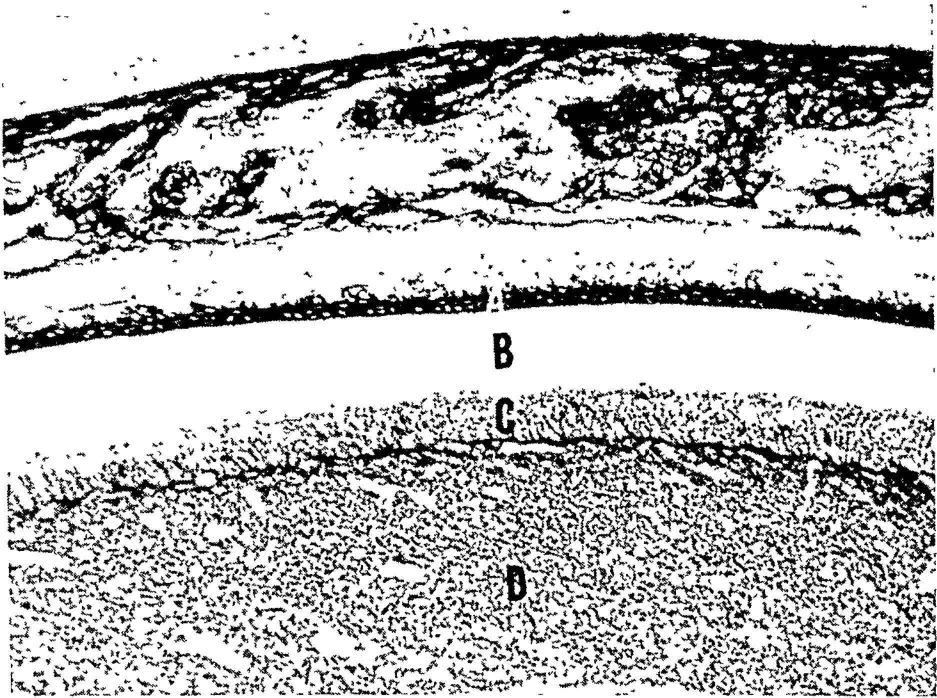


FIGURE 5 Incisor tooth of 3-day-old hamster showing strong alkaline phosphatase activity of stratum intermedium (A), odontoblasts (C), and pulp (D) The ameloblasts (B) are unstained Noncoupling technique as in FIGURE 2. ×143.

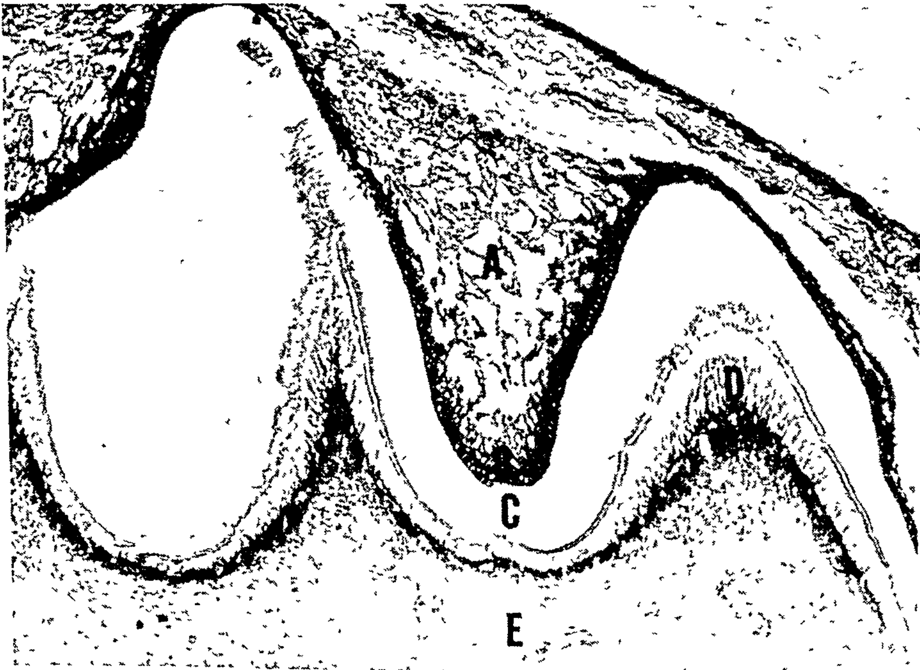


FIGURE 6 Alkaline phosphatase activity of unerupted molar from 2-day-old hamster. Naphthol AS-TR phosphate The stellate reticulum (A) and stratum intermedium (B) are strongly active (C) Ameloblasts, (D) odontoblasts, and (E) pulp. ×80

addition to being highly insoluble, is fluorescent under ultraviolet light. These procedures represent innovations in enzyme histochemistry. In general, the distribution of activity is similar to that reported with older techniques, except that the staining reaction was characterized by sharp localizations and absence of diffusion of dye to calcified matrices. Nuclear staining is never observed with these azo-dye techniques.

With regard to intramembranous bone formation, the endosteum and periosteum stained intensely (FIGURE 1, *top*). Osteoblasts and their cytoplasmic processes stained intensely (FIGURES 2 and 3, *bottom*), as did osteocytes. Osteoclasts were invariably unstained, although they might conceivably exhibit low alkaline phosphatase activity.

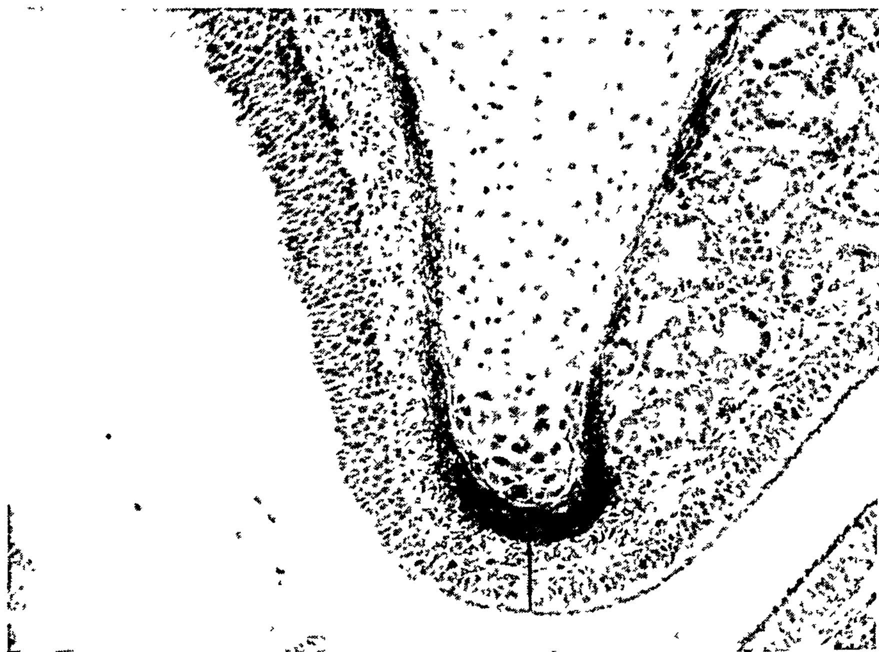


FIGURE 4 Nasal cartilage of 3-day-old hamster, showing activity of perichondrium and early center of ossification Nuclear stain is hematoxylin. Naphthol AS-TR phosphate. ×143.

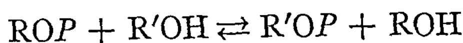
Sites of intracartilagenous calcification showed strong activity in the hypertrophic cells and in both cells and matrix of the provisional zone of calcification. This is essentially similar to the findings of Greep *et al.*[10] In addition, the perichondrium of both articular and hyaline nasal cartilage stained intensely (FIGURE 4).

The developing teeth revealed strong activity of the stratum intermedium and stellate reticulum (FIGURES 5 and 6). The ameloblasts were unstained (FIGURES 5 and 6) except in the area of the erupting incisor tooth The odontoblasts, associated Korff's fibers, and subjacent pulp were highly active (FIGURE 7).

When adenylic acid was used for the demonstration of 5-nucleotidase[1] the microscopic localizations were essentially the same as observed with the

Of interest is the fact that the *p*H optimum of AS-phosphates is around 8.3, which is somewhat lower than that observed with glycerophosphate.

Alkaline phosphatase may possibly function as a transferase in the calcification process according to the following reaction:[14]



$$\mathrm{RO}P + \mathrm{R'OH} \rightleftarrows \mathrm{R'O}P + \mathrm{ROH}$$

The nature of an acceptor is not known. In regard to endochondral ossification Gutman and Yu[16] have suggested a transphosphorylase system involving phosphopyruvate.

Acid phosphatase. There are few reports on the histochemical localizations of acid phosphatase in calcifying tissues. This is related to the relatively

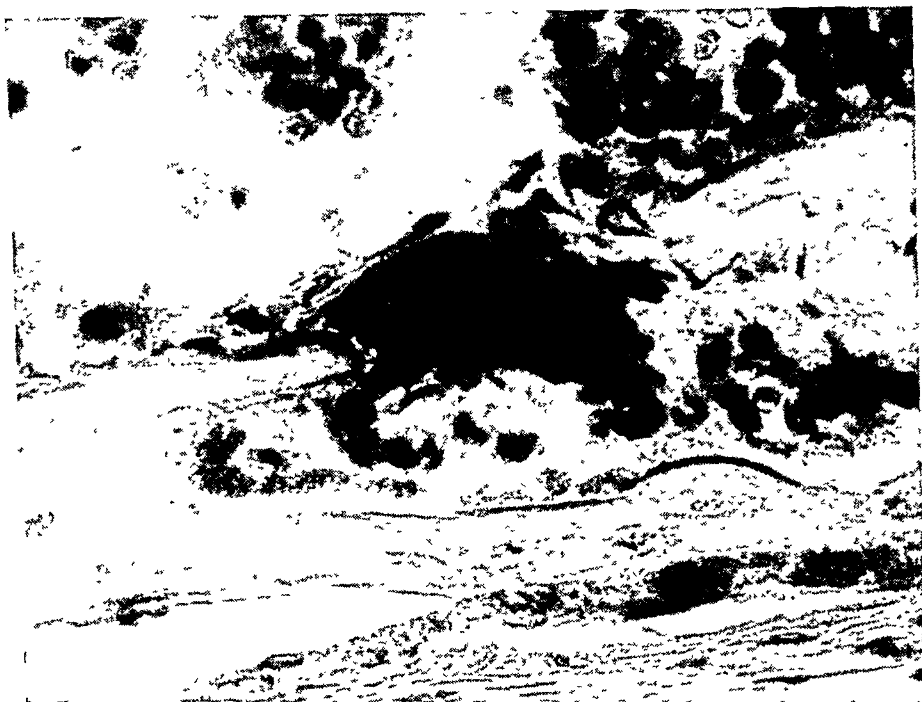


FIGURE 8 Acid phosphatase activity of osteoclasts of 3-day-old hamster knee joint Naphthol AS-BI phosphate, hematoxylin counterstain ×285.

low activity of the enzyme as compared with alkaline phosphatase and also to the poor reliability of older histochemical techniques.

When naphthol AS-phosphates are used in conjunction with frozen-dried tissues, consistently reproducible localizations of acid phosphatase activity are obtained in both soft and hard tissues.[16,17] High activity of osteoclasts was consistently observed (FIGURE 8). There was a positive reaction in the adjacent bone matrix. With certain substrate-diazonium salt combinations the reaction was observed primarily in the matrix.[18]

The biochemical function of acid phosphatase has not been elucidated. Roche[13] comments that most tissues endowed with intense alkaline phosphatase activity are practically devoid of acid phosphatase activity. This observation is certainly confirmed by the mutually exclusive distribution of alkaline phosphatase (osteoblasts) and acid phosphatase (osteoclasts), as observed using

glycerophosphate and azo-dye methods. Cartilage is known to have high nucleotidase activity, and Reis[11] has related this to the calcification phenomenon. The use of adenosine triphosphate also revealed similar localizations. This substrate has been found in uncalcified cartilage by Albaum *et al.*[12] Gomori[1] (p. 187) points out that nonspecific alkaline phosphatase also hydrolyzes nucleotides; thus there may be some question of specificity with use of the aforementioned substrates.

The function of alkaline phosphatase in the calcification process has yet to be completely elucidated. Robison originally suggested that alkaline phosphatase affected release of phosphate ions and thus exerted a direct effect



FIGURE 7. Higher magnification showing alkaline phosphatase activity of odontoblasts and Korff's fibers of incisor tooth from 4-day-old hamster. (E) Enamel matrix, (D) dentin matrix, and (P) pulp Naphthol AS-TR phosphate. ×300.

upon the formation of bone mineral. These data have been reviewed and elaborated upon by Roche.[13] Unfortunately, an *in vivo* source of phosphate ester has not been found.

Of interest is the observation that alkaline phosphatase is present not only in cells (for example, in odontoblasts) directly associated with matrix formation, but also in the stratum intermedium. Phosphatase has been related to the passage of metabolites across cell membranes. Thus the stratum intermedium may exert a selective filtering effect upon metabolites to be incorporated in the enamel matrix.

Despite a rather high *in vitro* *p*H optimum for the enzyme, the histochemical reaction still proceeds at a high rate at physiological *p*H with the naphthol AS-phosphates as employed in the present study. It has been pointed out that the *p*H optimum is lowered when the substrate concentration is decreased.



FIGURE 10 Cytochrome oxidase activity in 3-day-old hamster knee joint Note staining of osteoclasts, as well as chondrocytes ×105.



FIGURE 11 Three-day-old hamster molar showing cytochrome oxidase activity of stratum intermedium (A), and odontoblastic layer (B) ×105.

the aforementioned AS-phosphate techniques. It may also raise the question as to the possible differentiation of osteoblasts into osteoclasts. Ham[19] feels that osteoclasts arise from undifferentiated connective tissue cells. He also quotes Haythorn to the effect that osteoclasts may be classified as foreign-body giant cells. Of interest is the fact that foreign-body giant cells show high acid phosphatase activity. Gaillard[20] points out that, in tissue culture, osteoclasts always accompany the process of lacunar resorption. He suggests further that a "secretory" function of the osteoclasts is related to physiological resorption, but offers no confirmatory evidence.

Aminopeptidase. This enzyme can be visualized through the use of an azo-dye method employing *l*-leucyl-β-naphthylamide or *dl*-alanyl-β-naphthyl-



FIGURE 9. Aminopeptidase activity in developing (3-day) hamster molar (A) stratum intermedium, (B) odontoblasts ×143

amide.[21] Osteoclasts in the human showed an intense reaction, while those of other species were unstained in paraffin sections.[3] In the developing rodent dentition there was a distinct reaction of the stratum intermedium and odontoblasts (FIGURE 9). There was some staining of the periosteum Chondrocytes as well as the perichondrium were also reactive.

The biochemical function of aminopeptidase is not clear. It is of interest, however, that macrophages show intense activity, as do certain sites associated with breakdown of connective tissue. Hancox reported that osteoclasts *in vivo* have strong proteolytic abilities and are capable of liquefying a plasma clot.[22]

Cytochrome oxidase and succinic dehydrogenase These two enzymes will be considered jointly because of their almost identical localization in bone and tooth structures. Cytochrome oxidase, a major respiratory enzyme, has been

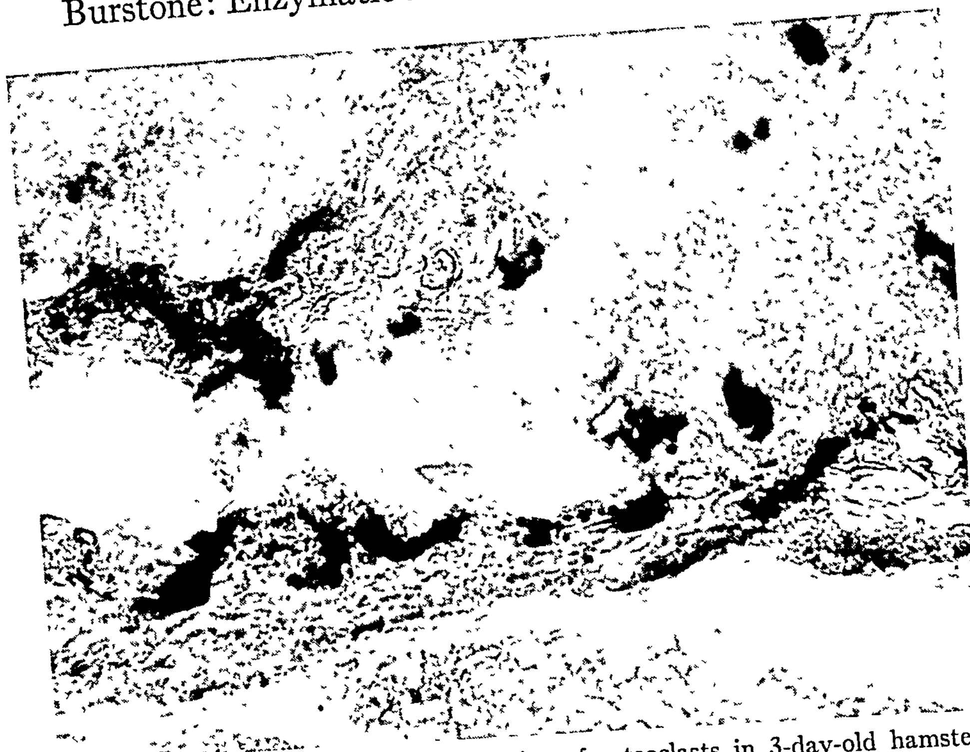


FIGURE 13. Succinic dehydrogenase activity of osteoclasts in 3-day-old hamster head intramembranous bone. ×223.

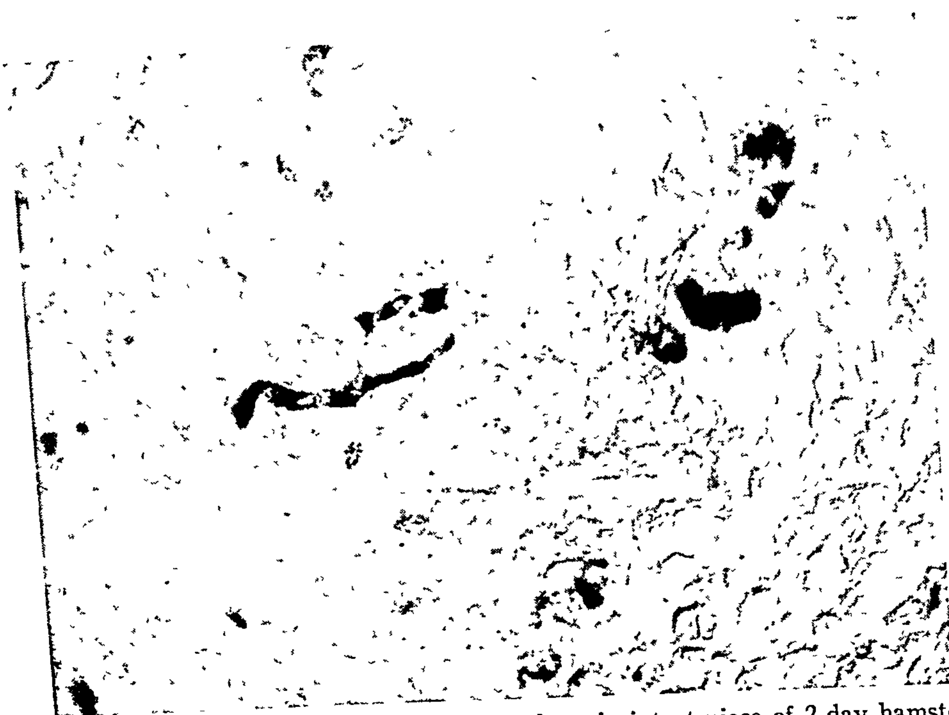


FIGURE 14. Succinic dehydrogenase of osteoclasts in intact piece of 2-day hamster calvarium. ×210

visualized in the past by the so-called Nadi reaction of Ehrlich. This reaction, when employed for histochemical purposes, is not entirely satisfactory because of poor localizations and because of the tendency for the reagent to auto-oxidize. New techniques for the visualization of this enzyme were employed to delineate more accurately the sites of cytochrome oxidase in calcifying tissues. These techniques employ postchelation of the indoaniline or azomethine dye with metals, which enhances the permanency of the preparation.[23,24]

The *p*-aminodiphenylamine (*N*-phenyl-*p*-phenylenediamine) was used with the three following naphthol or methylene compounds as substrates: (1) 1-hydroxy, 2-naphthoic acid; (2) 1-$OHC_{10}H_6$-2-$CONHC_{18}H_{37}$; and (3) α,α'-terephthaloylbis (5-chloro-2,4-dimethoxy-acetanilide).

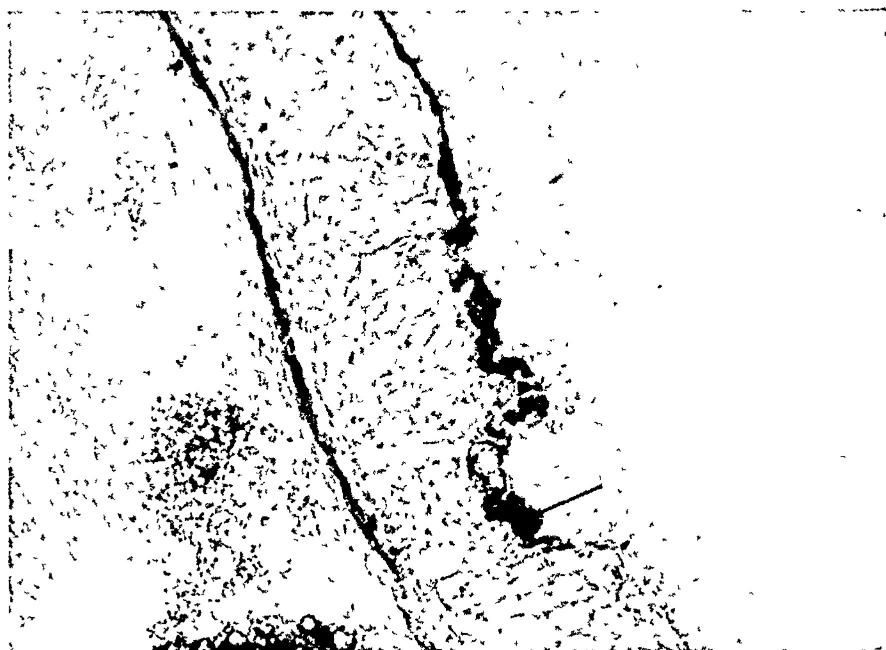


FIGURE 12 Succinic dehydrogenase activity of cartilage of 3-day-old hamster. Note activity of perichondrium and osteoclasts associated with destruction of cartilage ×210

Since cytochrome oxidase activity of calcifying tissues is lower than that of muscle or brain, the reaction was augmented by the addition of cytochrome *c* (10 to 20 mg. per Coplin jar). The incubation time was approximately 30 min.

Both osteoclasts and osteoblasts showed activity, the staining of the former predominating (FIGURE 10). Pieces of calvarium from 2-day-old hamsters were sufficiently thin to observe definitive staining of osteoclasts. The stratum intermedium of both molar and incisor teeth, the ameloblastic and odontoblastic zones were active (FIGURE 11). Cartilage revealed a perichondrial reaction, as well as a strong staining of hypertrophic cells (FIGURE 10).

Succinic dehydrogenase was demonstrated by the use of several tetrazoles. These included neotetrazolium,[25] 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride,[26] 3-α-naphthyl-2,5-diphenyltetrazolium chloride, nitro

tion of osteoclasts was striking (FIGURES 12, 13, and 14). The marked oxidase and dehydrogenase activity of osteoclasts certainly suggests that these cells possess high metabolic activity. Characteristic activity was also observed in the stratum intermedium and odontoblastic zones of both molar and incisor teeth (FIGURE 15).

Summary

TABLE 1 indicates the range of higher activity of different elements of developing hard tissues of the hamster, rat, and mouse. With prolonged incubation periods, sites of very low activity may be revealed. Of interest is the fact that sites of high activity involving a number of enzymes are to be found in the same structures (for example, the stratum intermedium and odontoblasts). This finding suggests high metabolic activity of the aforementioned cells.

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blue tetrazolium,[27] and nitroneotetrazolium.[28] Substrates were dissolved in ethanol or N,N'-dimethylformamide and used at pH 7.4 (Tris buffer) in the presence of sodium succinate. In cartilage the distribution of activity was similar to that of cytochrome oxidase (FIGURE 12). However, there was only slight staining of osteoblasts, ameloblasts, and odontoblasts. The reac-

TABLE 1
ENZYME ACTIVITY OF CELLS ASSOCIATED WITH BONES AND TEETH

		Alkaline phosphatase*	Acid phosphatase*	Aminopeptidase†	Cytochrome oxidase†	Succinic dehydrogenase†
Bone	Osteoblasts	++	0	+	+	+
	Osteocytes	++	0	+	+	+
	Osteoclasts	0	++	?	++	++
Cartilage	Active perichondrocyte	++	0	++	++	++
	Resting chondrocyte	0	0	+	+	+
	Hypertrophic chondrocyte	++	0	+	+	+
Tooth	Stellate reticulum	++	0	+	+	+
	Stratum intermedium	++	0	+	+	+
	Ameloblasts (molar)	0	0	0	0 or +	0
	Odontoblasts	+ or ++	0	+	+	+

* Frozen-dried paraffin embedded tissues.
† Fresh-frozen tissues
Key: 0 = no staining, + = less active; ++ = more active

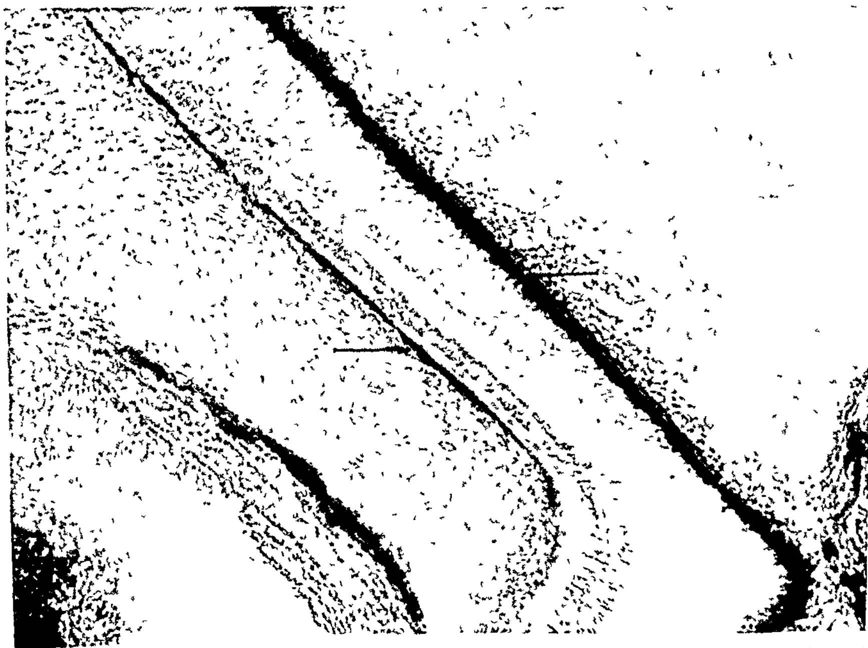


FIGURE 15. Succinic dehydrogenase activity of stratum intermedium and odontoblastic zones of 2-day hamster molar. ×143.

THE SEQUENCE OF EVENTS IN OSTEOGENESIS AS STUDIED IN POLYETHYLENE TUBES

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Histologically and physiologically, the periodontium may be considered to have the structure of a joint that attaches the tooth to the maxilla or mandible. In the regeneration of joint structure the healing process is osteogenic and thus, comparable in many respects to the healing of a fractured bone.[1] Regeneration may not be induced, however, owing to the organization of the blood clot through the fibrous rather than the osteogenic pathway. The question arises: What are the factors that determine an osteogenic rather than a fibrous repair? From a clinician's viewpoint the problem can be stated: How can an osteogenic process be induced where normally fibrous repair occurs? This problem has been investigated in one form or another for a long time. For example, most textbooks on bone describe the famous controversy that raged for more than two hundred years about the role of one element, the periosteum, in osteogenic repair. We shall see later that this problem is probably still with us, as the experiments I am reporting today appear to cast doubt on the explanation that abated the controversy.

In one study investigating the requirements for osteogenic repair, gaps of approximately 15 mm. were prepared in the fibulae of a dog by the removal of a section of both bone and periosteum. The two fragments of the fibula were completely separated from each other. As would be expected, healing of this lesion normally occurs through the fibrous pathway and results in a fibrous union. One fibula acted as a control and the other as a test. One of the methods found to induce an osteogenic repair, indicated by a bony union, was that of bridging the gap with a polyethylene tube, in some cases filled with autogenous cancellous grafts, that is, small chips. Care was taken to make sure that the polyethylene tube was filled with blood. Progress in healing was followed radiologically and, in some cases, histologically. FIGURES 1 and 2 show radiographs of a case in which the polyethylene tube alone was used.

The X ray shown in FIGURE 1*a* was taken at 18 days after operation. The fibula was two separate pieces of bone some distance apart. The polyethylene tube was used on bone on the left, and the bone on the right was the control. At 27 days an interesting phenomenon appeared (FIGURE 1*b*). A tip of bone was growing on the ends of the bony fragments, most noticeably on the distal fragment. At 42 days the growing tip had nearly crossed the gap (FIGURE 2*a*). The bony union in FIGURE 2*b* indicates that in some way the tube caused an osteogenic rather a-fibrous repair. A fibrous repair occurred in the control.

Cellular invasions of the blood clot in the polyethylene tube started from both cut ends of the bone and proceeded gradually down the tube, meeting near the center of the gap. Histological sections made on the twenty-eighth day of a case in which the gap was filled with autogenous cancellous grafts showed that the blood clot in the tube at the center of the gap had not yet been invaded by cells, while bone had formed in the tube near the ends of the

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gap Thus a longitudinal section of the contents of the tube presented a continuous picture of the sequence of events from the cellular invasion of the blood clot to the formation of bone. The best section was photographed, field by field, from the center of the gap out to one end. In addition, a few photomicrographs of a case at 63 days were added. The representative fields in FIGURES 3 to 15 illustrate the sequence of events observed. The osteocytes and the endosteal cells of the grafts all died, as the tube prevented them from connecting with the circulation.

In FIGURE 3 is shown the blood clot in the center of the gap that was as yet

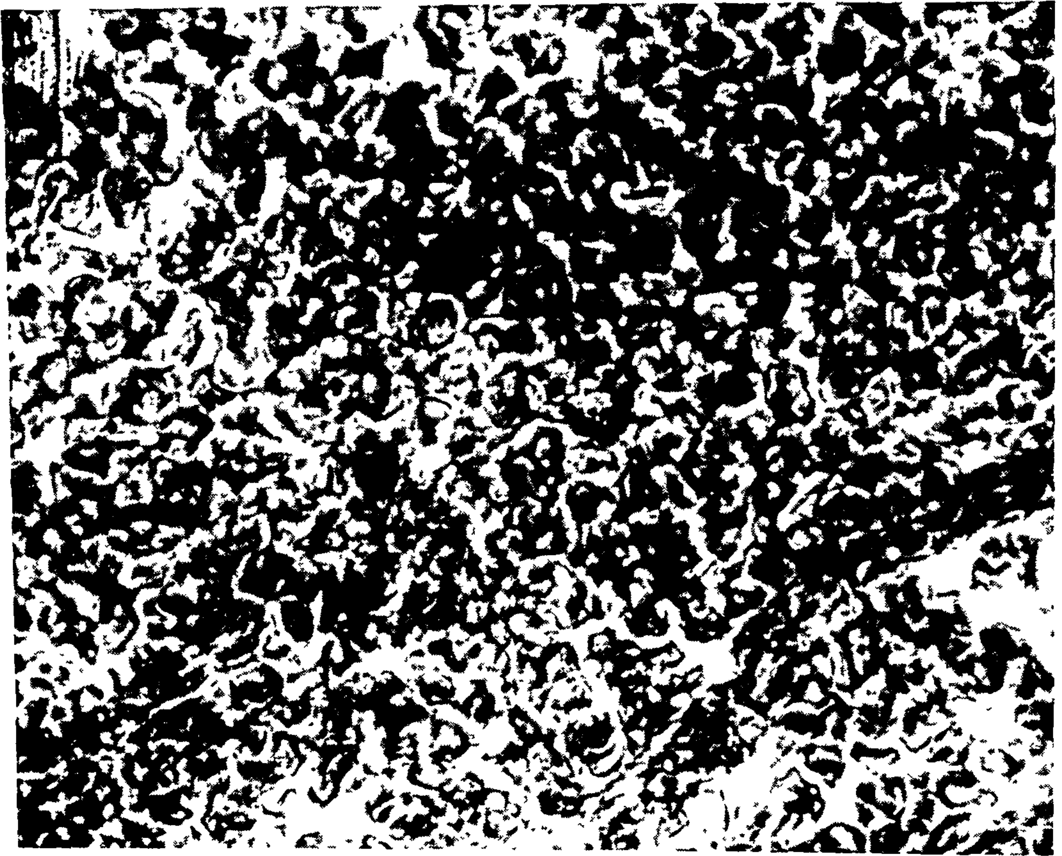


FIGURE 3

uneffected by the growth process. In FIGURE 4, part of a bone graft may be seen lying in the blood clot. In one sense this field represents the first day of the healing process. However, the grafts had been lying in serum for 28 days. Had there been any change in the grafts during that period? A little further down the tube (FIGURE 5*a*) we see the beginning of the process; that is, cells are growing in the blood clot, an early stage in healing FIGURE 5*b* shows the blood clot becoming more organized.

In FIGURE 6*a*, the blood clot is now completely organized by a relatively undifferentiated mesenchymal tissue. The same tissue under higher magnification is shown in FIGURE 6*b*.

FIGURE 7*a* depicts a graft lying in this undifferentiated tissue. There seems



FIGURE 1.

FIGURE 2.



FIGURE 5.

to be no reaction in the tissue to the graft; the grafts have a comparatively smooth surface. The surface enclosed in the rectangle, however, appears different. A higher magnification of this surface is given in FIGURE 7b, in which it is apparent that the striated appearance of the margin occurs where the direction of the collagen fibrils of the bone is roughly at right angles to the surface. The fibrils in the bone appear to be continuous with those in the striated border. Also, the cells and the reticular fibrils of the new tissue appear to have grown into the striated border. The grafts had been in serum or in the tissue for 28 days; hence this development is consistent with the hypoth-

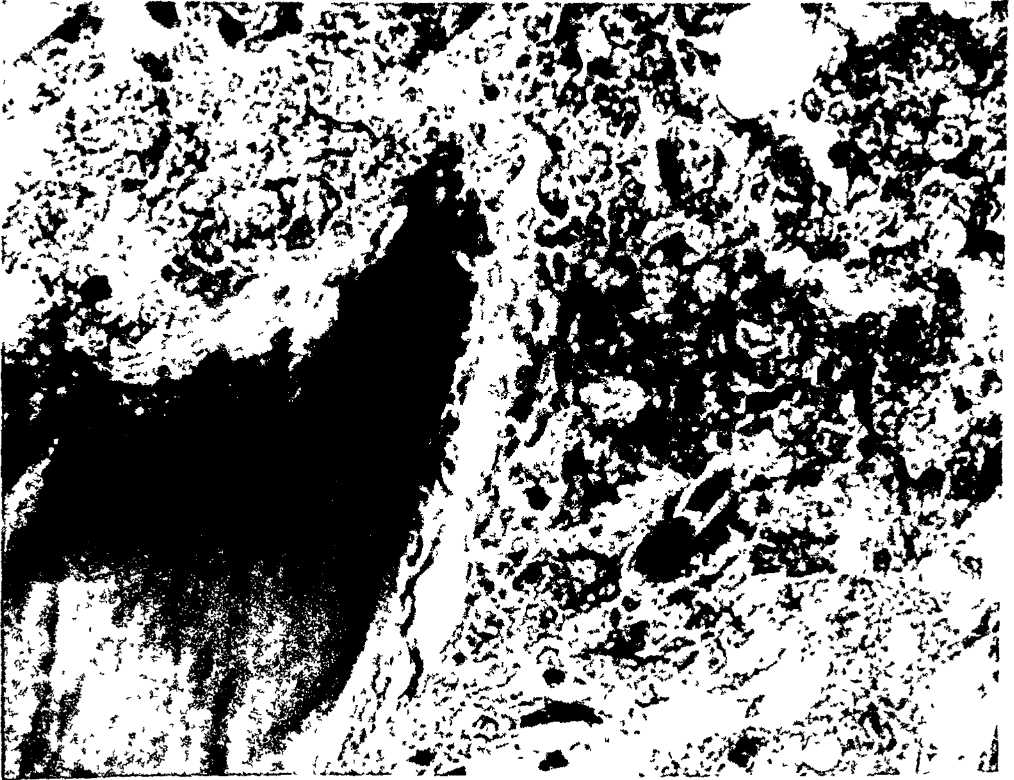


FIGURE 4

esis that resorption of the graft had occurred during that time. Furthermore, the striated borders appear to result from the mineral contents of the bone dissolving before the collagen fibrils dissolve, leaving the latter to project a short distance from the surface of the bone. The fibrils in turn seem to resorb at the free ends at about the same rate as the mineralized element dissolves, so that the striated borders never become very thick.

The next stage FIGURE 8 seems to be the differentiation of osteoclasts. The moth-eaten appearance of the grafts, caused by osteoclastic resorption, is distinctly different from the smooth appearance of the grafts before osteoclasts appeared. The evidence suggest two types of resorption, the first being the result of the grafts dissolving in serum and, second, the result of the action of osteoclasts.



Figure 7.



FIGURE 6



FIGURE 9.

An example of the ciliated border sometimes seen in osteoclasts, described by Ham[2] and Mallory,[3] is depicited in FIGURE 9*a*. Ham advanced the concept that the ciliated border is caused by the resorption of the bone and fibrils of the grafts shown in FIGURE 7*a*. FIGURE 9*b* shows a section of ciliated border treated with Mallory's connective tissue stain. It appears that the ciliated border is the result of the growth of protoplasmic extentions of the osteoclast into the striated border.

In FIGURE 10*a*, the osteoclasts have disappeared. The resorptive stage is over and the deposition of bone has begun. With higher magnification (FIGURE

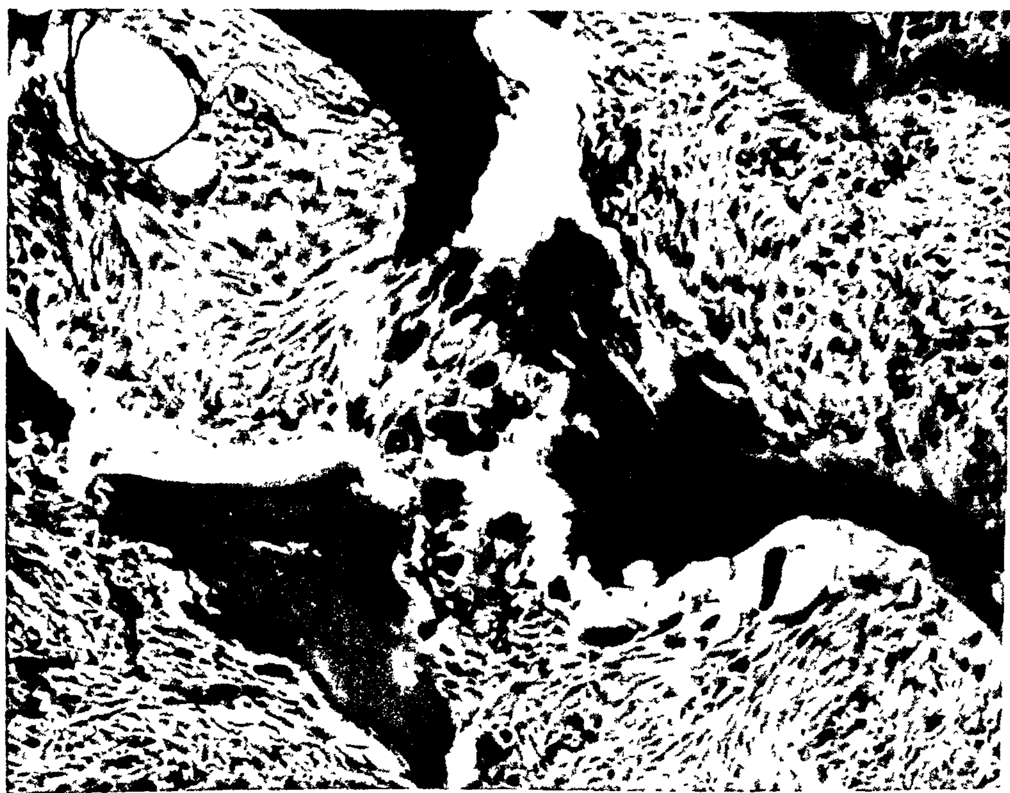


FIGURE 8.

10*b*) the contrast between the remnants of the graft and the new bone is quite apparent. There are empty lacunae in the old bone and deep-staining osteocytes in the new bone. The old bone is lamellar bone and the new bone is woven or immature bone.

In FIGURE 11 we can review the sequence of events leading up to this stage. the organization of the blood clot, the resorption of the graft on the left, and the apposition of woven bone. In this stage, the cells that had been differentiating to osteoblasts and forming bone are differentiating to chrondoblasts and forming cartilage. In FIGURE 12, osteogenesis has stopped and chrondogenesis is proceeding. FIGURE 13*a* suggests, however, that, while cartilage will form in this environment, it cannot be maintained. The cartilage has

died, calcified, and is being resorbed. FIGURE 13*b* is a higher magnification of the cartilage being resorbed, showing, in particular, a large osteoclast.

The formation of new bone on the remnants of the cartilage (FIGURE 14) provides a contrast between the dead cartilage cells and the new osteocytes This phase of repair appears to be similar to that occurring in the lengthening of a diaphysis. The resorption of dead calcified tissue, in this case dead cartilage, followed by apposition on the remnants appears to be quite similar to an earlier phase of the process, that is, the resorption of the dead bone grafts followed by apposition on the remnants. In both cases resorption of calcified tissue is followed by apposition. In FIGURE 15, the new bone deposited on the remnants of the cartilage must have been resorbed, as the cartilaginous cores have disappeared. However, the presence of osteoclasts on the left and osteoblasts on the right indicates that the two processes, resorption and apposition, are still going on.



FIGURE 11

Thus far we have simply noted what occurs in one phase of osteogenic repair. However, as Claude Bernard states, "By simply noting facts we can never succeed in establishing a science. Pile up facts as we may, we shall be none the wiser To learn we must necessarily reason about what we have observed."[4] Therefore, we now develop working hypotheses about the nature of the process underlying the observed events.

a

b

FIGURE 10.

a

FIGURE 13.

b

We must be quite clear about the process we have been studying, which is that phase of osteogenic repair in which continuity in a bone is restored by woven or immature bone. I have been referring to this as the pathological phase. Once continuity is established, the woven bone is replaced by lamellar bone in what we refer to as the remodelling process, the adaptation to function, or the maintenance procedure. This process continues throughout life as bones continually adapt to the burdens they have to bear. I have been referring to this as the physiological phase. It is important to make a definite distinction between the two phases in osteogenic repair.

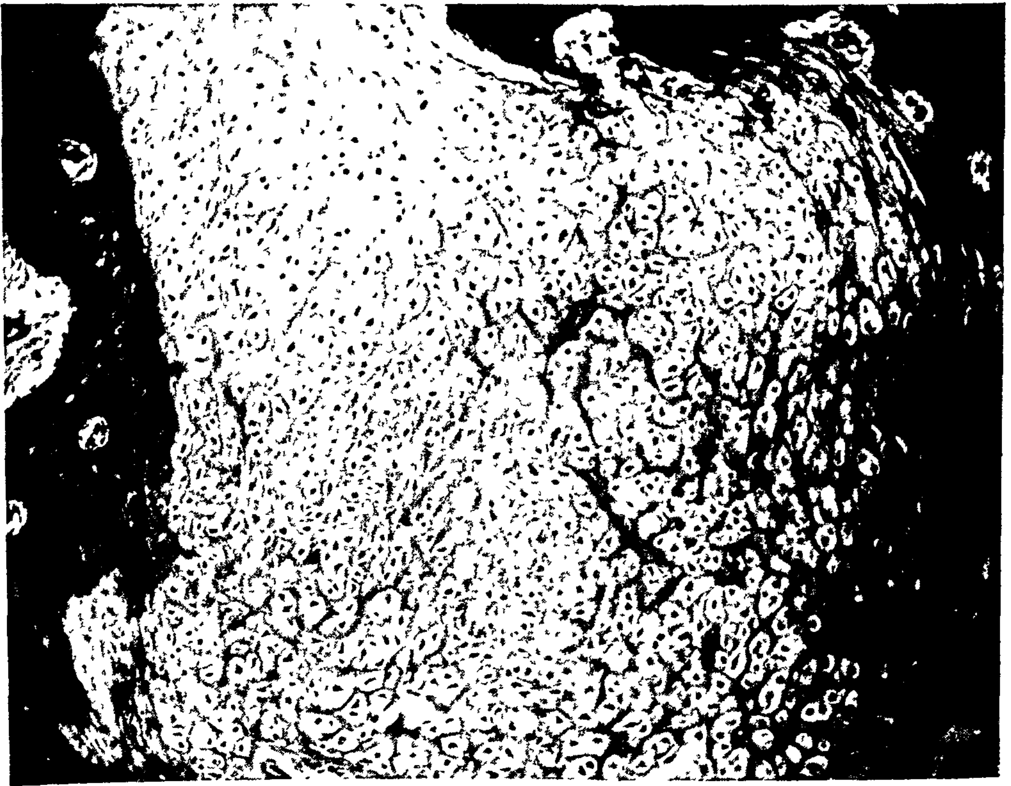


FIGURE 12.

Consider the sequence of events following the organization of the blood clot by the undifferentiated tissue. Osteoclastic resorption is followed by the formation of bone and apposition; apposition then stops and cartilage forms, calcifies, and dies. Resorption recurs, followed by a wave of apposition; subsequently, resorption of bone with its cartilage cores is followed by apposition; resorption and apposition continue. It appears that the process is a complicated one, consisting of building, tearing down, and rebuilding, until the end result, the re-establishment of continuity, is attained.

From these and other observations I have developed a concept of the nature of the process. I believe that each stage prepares for and initiates the following stage, in a type of chain reaction. One outstanding feature of the process is

fails, and we have another resorption-apposition cycle. As soon as continuity is established, the pathological phase is over. In the physiological phase that follows, the woven bone is resorbed and replaced by lamellar bone.

Dead bone grafts are known to aid osteogenesis, and they do so, perhaps by supplying calcified material to be resorbed, thus promoting initial bone formation farther away from the bone surface. Grafts may aid osteogenic repair in other ways, as well.

In this paper I have referred to another interpretation of our observations, namely that bone and cartilage may form in an environment in which they

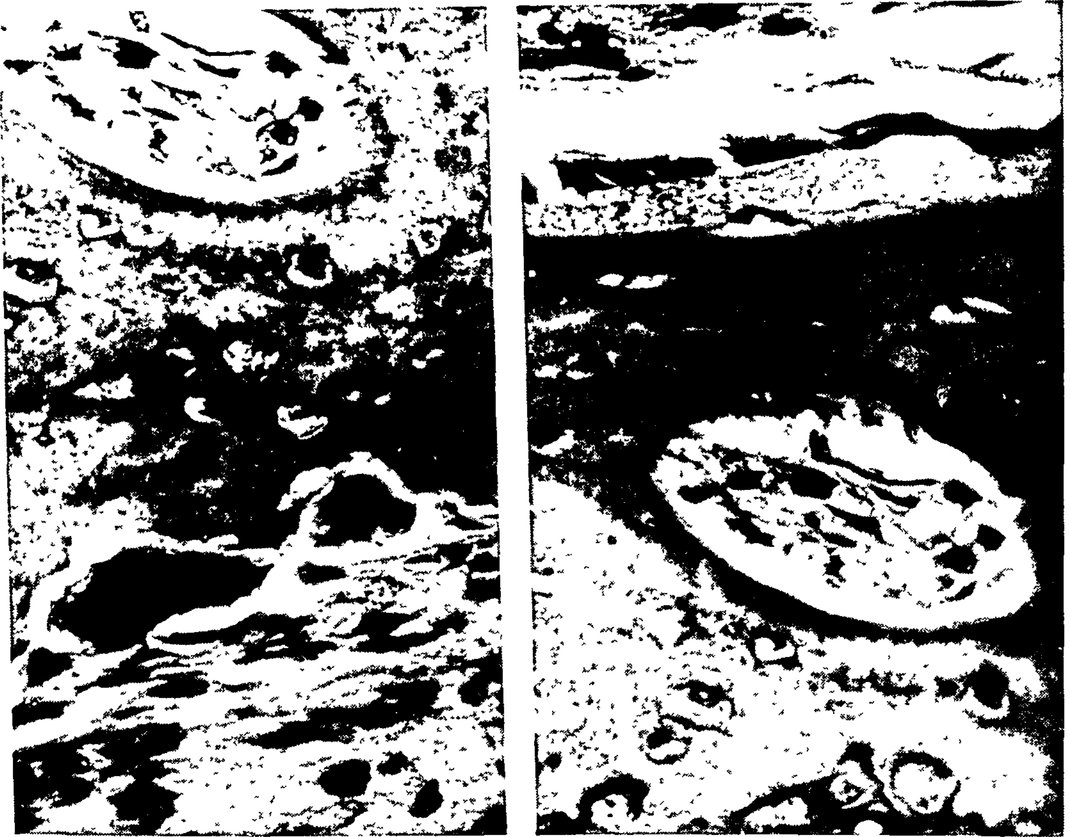


FIGURE 15

cannot be maintained. Preliminary studies suggest that this is true of connective tissue also. If this is the case, the mechanisms of tissue formation differ in some important respects from those involved in maintenance. This consideration is relevant to the study of the physiology of skeletal tissues.

In conclusion, I summarize the above interpretations of the process of re-establishing continuity in bone as follows:

Each stage in the process is prepared for and initiated by the preceding stage.

The formation of bone is a self-limiting process, requiring the resorption of calcified tissue for initiation of another wave.

Cartilage and bone may form in an environment in which they cannot be maintained, suggesting that the processes of formation and maintenance differ in some important respects.

the cyclic recurrence of the resorption and apposition mechanisms. If the apposition of bone were self-limiting, that is, if the formation of bone itself changed the environment in such a way that bone formation stopped and did not recur until initiated by the resorption of a calcified tissue, a cyclic process would result.

This hypothesis can be illustrated by applying it to the healing of a lesion that has been extensively studied, for example, the repair of a fracture. The goal is re-establishment of continuity

The osteocytes and the covering and lining cells near the line of fracture die.

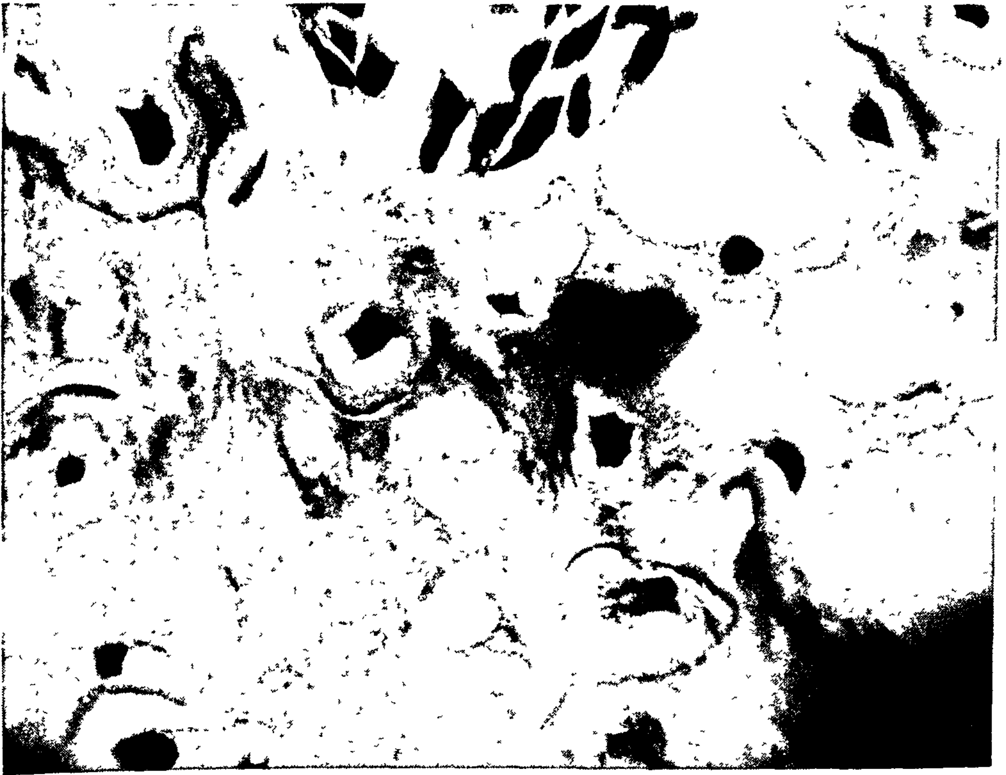


FIGURE 14

The blood clot is organized by proliferation of the covering and lining cells a little farther away from the line of fracture, first around the dead bone and then in the gap between the fragments. We have seen that this situation leads to resorption followed by a wave of apposition If continuity is established, the pathological phase is over. If, however, continuity is not established, the self-limiting feature causes bone formation to cease, but produces conditions initiating the formation of cartilage. It appears that, while cartilage can be formed in this environment, it cannot be maintained in it. It dies, becomes calcified, is resorbed, again producing conditions that initiate another self-limiting wave of bone formation. By this time a considerable amount of bone is formed in the unorganized fashion of woven bone The formation of bone on bone causes some of it to die because the nutrition of some of the osteocytes

HYDROLYTIC ENZYMES IN PERIODONTAL TISSUES*

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The purpose of this investigation was to explore and describe some of the hydrolytic enzyme activities present in human "normal and abnormal" periodontal tissues. The philosophy underlying and motivating this approach developed over a period of years and was informally projected in 1953 with my colleague Bertram Eichel who, at that time, was a member of the School of Aviation Medicine, Randolph Air Force Base, Texas. Simply stated, it was and still is strongly felt that really fundamental understanding of the problems of the oral cavity could be elucidated best by investigating a broad spectrum of enzyme activities as they reflect the metabolic state of both normal and abnormal tissues.

Previous studies with whole human saliva had furnished leads indicating that certain hydrolytic enzyme relationships or patterns existed that could be used statistically as potential diagnostic criteria. Lisanti[1] suggested that hyaluronidase activity in whole human saliva could be correlated with the presence of periodontal disease and caries. Later, Lisanti and Mahler[2,3] reported that patients involved with periodontal and upper-respiratory diseases exhibited a marked increase in the salivary hyaluronidase titer. Subsequently, Lisanti and Chauncey[4] confirmed and extended these findings to include a spectrum of hydrolytic enzymes. Of 10 enzymes studied, 3 showed a correlation pattern with the occurrence of periodontal disease and 3 with the presence of dental caries. Aspects of the latter findings will be examined here as *Phase 1* of this report.

Phase 1

Experimental. Ten hydrolytic enzymes were identified in human saliva: acid phosphatase, alkaline phosphatase, total esterase, cholinesterase, lipase, sulfatase, beta-D-glucuronidase, galactosidase, hyaluronidase, and lysozyme.[5,6] A high degree of correlation was noted for hyaluronidase, lipase, cholinesterase, and beta-glucuronidase when dental caries and/or periodontal disease were present in the mouth of human subjects. The activities of each enzyme were arranged in rank order and medians were determined. The medians were used as a break point Those below the break point were considered low activities, and those above were considered high activities. The subjects were classified by means of clinical and radiographic procedures and distributed into 5 groups: (1) normal—apparently free of dental caries and periodontal disturbance, (2) dental caries present and no periodontal disease, (3) periodontal disease and free of dental caries, (4) both dental caries and periodontal disease, and (5) edentulous.

* The work reported in this paper was supported in part by the Office of Naval Research, Department of the Navy, Washington, D.C., and in part by the Warner-Lambert Pharmaceutical Company, Morris Plains, N J.

One way in which dead bone grafts aid osteogenesis is by providing calcified tissue to be resorbed.

Dead bone grafts may be dissolved by processes other than that involving the osteoclasts.

The observation that bone can be induced to grow into a space is of particular interest to dentistry. A tube of polyethylene or some other material appears to be a useful tool for studying the process.

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are these enzymes? (2) Do the oral tissues contribute to the pool of salivary enzymes? (3) To what extent can these enzymes further reflect disease states? The first question was partially answered by Lorina and Lisanti,[7] Lorina *et al.*,[8] and Mahler and Lisanti,[3] who have shown that certain types of oral microorganisms are capable of elaborating beta-glucuronidase and hyaluronidase. These microorganisms were obtained from the oral cavities of subjects giving evidence of dental caries and periodontal disease. The organisms have been isolated from saliva, carious lesions, affected gingival tissue, and tooth scrapings.[2,3]

Schultz-Haudt and his associates have confirmed some of these findings. They have isolated hyaluronidase-producing bacteria[9] and beta-glucuronidase-producing bacteria[10] from gingival crevices and have demonstrated hyaluronidase activity in filtrates prepared from "gingival deposits."[11]

With reference to the second and third questions posed above, the human gingiva was selected for direct study employing histochemical methods for the disclosure of hydrolytic enzyme activities. These studies have been summarized by Lisanti.[12] The preliminary aspects of the latter study constitute the second phase of this paper.

Phase 2

Handling of tissues and histochemical methods. Twenty-five biopsy samples of human gingiva were studied as soon as possible following gingivectomy. The gingivae were pretreated prior to surgical removal. One interdental papilla, the smallest taken for study, was removed from each biopsy specimen. Tissue sections, approximately 25 μ thick, were prepared on the freezing microtome. The orientation of each tissue sample was such that it was possible to obtain sections that could be related with the portion of the gingiva that approximated the root surface and the gingival and epithelial margin away from the tooth, the crest of the interdental papilla, the approximate depth of the inflammatory process, and an indication of the pocket depth.

In order to determine the degree of histopathology in each tissue section the following procedure was adopted. Serial sections were prepared and evaluated histochemically in a typical sequence as follows:

Sections 1 and 2 were stained with hematoxylin and eosin for histopathological evaluation, 3 and 4 for alkaline phosphatase,[13] 5 and 6 for beta-D-glucuronidase,[14] 7 and 8 for galactosidase,[15] 9 and 10 again with hematoxylin and eosin, 11 and 12 for glucosidase,*[16] 13 and 14 for esterases,[17] 15 and 16 for acid phosphatase,[18] 17 and 18 for cholinesterase,[19] and 19 and 20 once again with hematoxylin and eosin. This procedure permitted examination of the degree of inflammation in the tissue based upon histopathological examination of the beginning, middle, and end of the series. At no point in the histochemical disclosure and localization of the enzyme activities was any section more than 5 serial sections away from a histopathological reference point.

Results. The results described below have been limited to descriptions of

* The histochemical method employed here was a direct adaptation from the colorimetric method of assay for glucosidase.

The patterns of enzyme activities recognized and the relationships found are given in TABLE 1. In pattern A the enzyme activities of human saliva that correlated with dental caries in the mouth showed high cholinesterase, low lipase, and low hyaluronidase activity. In pattern B the enzyme activities of human saliva that correlated with periodontal disease in the mouth exhibited low cholinesterase, high lipase, and high hyaluronidase activity. Examination of the data for groups 1 and 5 (individuals without decay or periodontal disease) showed that 10 subjects yielded an enzyme activity picture that correlated with pattern A and 10 subjects with pattern B For group 2 (individuals having dental caries) 9 subjects corresponded with pattern A, and 3 subjects with pattern B. The periodontal disease subjects (group 3) showed 8 individuals who had an enzyme picture that corresponded with pattern B and only 2 subjects with pattern A. Group 4 (subjects showing evidence of both dental caries and periodontal disease) yielded evidence that 8 of these subjects corresponded to pattern B and 4 subjects to pattern A It was of interest that 75 per cent of the subjects having dental caries only and 80 per cent of the subjects having periodontal disease only could be identified in a mixed population of 54 subjects. For those individuals who had both dental caries and periodontal disease the enzyme pattern for periodontal disease predominated (62 per cent) over the enzyme pattern for tooth decay (38 per cent). The latter group also gave evidence for a high correlation with the presence of above average beta-D-glucuronidase activity in whole human saliva

TABLE 1

HYDROLYTIC ENZYME PATTERN ACTIVITIES OF WHOLE HUMAN SALIVA IN NORMAL, ENDENTULOUS, CARIES AND PERIODONTALLY INVOLVED SUBJECTS

Enzyme pattern	Groups 1 and 5*		Group 2		Group 3		Group 4	
	No caries, no periodontal disease		Caries		Periodontal disease		Caries and periodontal disease	
	No	%	No	%	No	%	No	%
A High cholinesterase Low lipase Low hyaluronidase	10	50	9	75	2	20	4	38
B Low cholinesterase High lipase High hyaluronidase	10	50	3	25	8	80	8	62
Beta-D-glucuronidase	–		–		–		+	

* The edentulous subjects were classed with the normals; that is, those free of caries and periodontal disease

These findings raised several questions that, once answered, could clarify the role of the hydrolytic enzymes in periodontal disease and other oral disease conditions. These were posed as follows: (1) In which components of saliva

Cholinesterase, pseudo (FIGURE 1g). Cholinesterase activity was characterized by the formation of purple stain localized principally around blood vessels and in association with inflammatory cell masses. There was an indication that some cholinesterase reaction occurred in the epithelial layers. The latter, however, is open to question owing to the lack of specificity for the substrate employed in the enzyme reaction mixture.

Summary

The statistical treatment of the data obtained from the hydrolytic enzyme assays performed with whole human saliva resulted in the development of enzyme activity patterns associated with dental caries and periodontal disease. The enzyme pattern related to dental caries was found to be high cholinesterase, low lipase, and low hyaluronidase; the pattern correlated with periodontal disturbances was low cholinesterase, high lipase, and high hyaluronidase. For those subjects exhibiting both dental caries and periodontal disease a high correlation existed, with above average beta-D-glucuronidase activity in their whole saliva.

The general impressions of the sections prepared from the 25 human gingiva biopsy samples are:

Alkaline phosphatase appears to be indicative of cellular proliferation. It is present in the sites of normal cellular multiplication and is associated with inflammatory cellular infiltration.

Beta-D-glucuronidase was associated with the degree of inflammation and involvement in those areas surrounding the gingival pocket.

Galactosidase could be found only in low concentrations in the basal layer of the epithelium and in approximation to the periodontal pocket.

Glucosidase was found mainly in sites in and surrounding the involved periodontal soft tissues.

Acid phosphatase, esterases, and cholinesterase were always found in both normal and abnormal portions of the soft tissue adjacent to the involved teeth.

The histochemical findings disclose that the concentration of several of the hydrolytic enzymes was elevated in the areas and foci of inflammation.

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histochemical reactions in those sections giving evidence of inflammation.* Low-power photomicrographs of the staining reactions in the mucosal and submucosal tissues opposite the pocket areas are presented in the FRONTISPIECE, FIGURES 1*a* through *g*, *lower*).

Alkaline phosphatase (FIGURE 1*a*). The greatest concentration of alkaline phosphatase (purple color) was found in the connective tissue and in the basal layer of the epithelium. Relatively high concentrations of the enzyme surrounded the capillary and blood vessel walls in the submucosa. An occasional area of concentrated alkaline phosphatase activity was found in the region of accumulated inflammatory cells that would have approximated the pocket areas. The red purple color indicates lesser enzyme activity and was present throughout the remaining connective tissue and the superficial layers of the epithelium. Cabrini and Carranza[20] have studied alkaline phosphatase in normal gingiva. Other investigators have also described its localization in normal and pathological gingiva.[21-25]

Beta-D-glucuronidase (FIGURE 1*b*). The presence of this enzyme was detected by blue color formation. It was demonstrated in highest concentration in all areas of inflammation and in areas surrounding pocket lesions. Some of the enzyme could be detected in the outer areas of the pocket and in the tissue previously approximating the cementum. It appeared that the beta-D-glucuronidase could be only identified with inflammatory processes and the areas of infiltration.

Galactosidase (FIGURE 1*c*). This enzyme was identified by the lavender stain produced. The basal cell layer was weakly lavender-colored The prickle-cell layer exhibited very weak lavender stain. The enzyme was also detected in areas of inflammation or pocket formation and suppuration where bacterial invasion could have occurred readily.

Glucosidase (FIGURE 1*d*). The enzyme was identified by the production of violet color. Its pattern of occurrence paralleled that of galactosidase. A greater area of the interdental papilla is shown in the color plate

Total esterase (FIGURE 1*e*). The presence of esterase was established by the appearance of blue-green color. The esterase enzymes were found almost exclusively in the epithelial tissue. The color intensity appeared to increase from the basal cell layer toward the stratum granulosum Occasionally esterase activity could be detected in inflammatory areas surrounding a previously existing pocket.

Acid phosphatase (FIGURE 1*f*). The production of red-purple color indicates the presence of this enzyme. In contrast to alkaline phosphatase, most of the acid phosphatase activity was detected in the epithelial layers. The concentration of acid phosphatase increased from the basal cell layer to the superficial layer, the most intense deep red purple being localized in the keratinized surface layer. Some acid phosphatase activity was noted in the immediate area of inflammation approximating the root surfaces Recently, Cabrini and Carranza[26] have also studied the localization of acid phosphatase in human gingiva

* The greatly expanded investigation of the qualitative and quantitative aspects of hydrolytic enzymes in human "normal" and "abnormal" gingivae, supported in part by the School of Aviation Medicine, Randolph Air Force Base, Texas, from 1955 to 1957, will be reported elsewhere

BACTERIAL MECHANISMS IN PERIODONTAL DISEASE*

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Bacterial populations in close approximation to mucosal surfaces of the periodontium reach numbers at least as high as 10^{10} per gm. of materia alba, as indicated by unpublished data. It is axiomatic that a consideration of their metabolic potential and their capacity to disrupt normal tissue metabolism is essential to an understanding of the metabolism of the periodontium.

There is substantial but not conclusive evidence that the flora of the periodontium is responsible for inflammatory and suppurative phenomena in periodontal disease and that this same microbiota can produce comparable infective processes subcutaneously in experimental animals. Infection in guinea pigs, the animal of choice for such studies, is characterized by inflammation, necrosis, and suppuration microscopically comparable to the pathological changes in periodontal disease. Similar processes occur in man in infections attributable to human bites and in tropical ulcers, noma, and like conditions. A characteristic mixed flora, the so-called fusospirochetal complex, can be demonstrated in these infections of man and animals.

The flora is strikingly similar to that of periodontal disease. The infections respond, in general, to antibiotic therapy; similar favorable though transient results have been reported in the treatment of periodontal disease with antibiotics. (These observations are documented in a review by Rosebury, 1947.) On the basis of these considerations it has seemed profitable to use the guinea pig model as a means of studying the character of these infections with the expectation that at least some of its features would be applicable to the problem of human periodontal disease.

Initial studies were directed at reproducing the characteristic transmissible guinea pig infection with pure cultures. A number of guinea pig infections were studied exhaustively and, in general, were shown to be populated by spirochetes (*Treponema microdentium*), *Fusobacterium*, vibrios, spirilla, *Bacteroides* species, anaerobic streptococci and anaerobic diphtheroids (Rosebury *et al.*, 1950, Macdonald *et al*, 1954). We found, as earlier workers did, that individual cultures isolated from the lesion were not infective.

Cultures of 17 organisms, isolated from a single infection, were recombined by a method called the wheel-plate technique. Each culture was streaked, in turn, on a blood-agar plate from the periphery to a common center. Spirochetes were inoculated into the agar in the hub. After incubation, each streak could be examined to assure growth of each organism in pure culture and mixed growth of all organisms in the hub. Mixtures so prepared, when ground with broth and inoculated in 1-ml. amounts, regularly infected guinea pigs, producing the typical transmissible lesion (TABLE 1).

In further experiments in which the wheel-plate technique was used, organ-

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biochemical sense that they depend on the proliferation of a combination of organisms capable of elaborating a specific array of required but generally unidentified metabolites.

It is to this aspect of the problem that the remainder of this paper is directed. One of the 4 essential organisms was *B. melaninogenicus*, a black pigment-producing, strictly anaerobic, Gram-negative coccobacillus. This organism has been observed, principally in mixed culture, by many investigators. It is a regular inhabitant of the oral cavity, having been found in all of 200 cases (Burdon, 1928). It is also present in the gastrointestinal tract, the external genitalia, tonsils, and such pathological sites as lung abscesses and surgical infections (Weiss, 1943). We have also recovered it from normal excised mesenteric lymph nodes of man.

This organism was shown to be dependent for growth on a filtrable factor elaborated by a second member of the pathogenic quartet, the facultative

TABLE 2
PATHOGENICITY OF DIFFERENT RECOMBINATION MIXTURES IN GUINEA PIGS

Passage	Inoculum (ml)	Group of 16	Group of 4	Group of 12
1	Culture mash, 1	+*, +*, +, +, ++	+*, +*, +, +, +	±, ±, ±, ±, ±
2	Exudate, 0 1	+, +, ++*, ++	+*, +, +, +	
3	Exudate, 0 1	+, +, ++, ++	±, ±, ±, +*	
4	Exudate, 0 3		++*, +++	
5	Exudate, 0 3		++*, +++	
6	Exudate, 0 3		++*, ++	
7	Exudate, 0 1		+++, ++++	

Key: ±, small nodular lesion, little or no exudate; +, less than 1 in localized abscess; ++, localized abscess 1 in. or more in diameter; +++, localized abscess with extension, not fatal in 7 days; ++++, spreading infection fatal in 7 days or less Each symbol represents 1 guinea pig.
* Exudate used for passage.
Group of 16· total recombination; group of 4: assumed pathogenic fraction; group of 12: assumed nonpathogenic fraction.
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diphtheroid. It was evident that one function, if not the only function, of the diphtheroid was the elaboration of this essential nutrient, and that its nature seemed worth investigating.

An assay was developed, the basal medium for which contained 1.7 per cent trypticase (B.B.L.), 0.3 per cent yeast extract (Difco), 5 μg./ml. of hemin, and 0.1 per cent $NaHCO_3$ plus minerals. The heme was shown to be an absolute requirement for the strain of *B. melaninogenicus*. In practice, it was found convenient to use a peptone water filtrate of *Staphylococcus aureus* as the growth factor, and the above medium supported good growth when 1.0 per cent filtrate was added. Twice-washed cells of the *Bacteroides* strain were used as inocula, and the tubes were incubated usually for 4 days in 95 per cent H_2, 5 per cent CO_2 at 37° C. The growth response was read as optical density on a Beckman Model B spectrophotometer at a wave length of 690 mμ, the minimum absorption band for heme. A typical experiment is summarized in TABLE 3.

The factor was found to withstand boiling for 15 min. with little loss of ac-

isms were systematically deleted from the recombination mixtures to distinguish essential from nonessential components of this mixed anaerobic infection. A minimum combination of 4 organisms—2 *Bacteroides*, one of which was *B. melaninogenicus*, a motile Gram-negative anaerobe, and a facultative diphtheroid—was shown to produce the typical infection. When wheel-plate inocula of these 4 were compared to inocula of all 16,* or the presumably nonpathogenic 12, it was demonstrated that the combination of 4 produced infection comparable to the combination of 16 and that the combination of 12 was

TABLE 1
PATHOGENICITY OF RECOMBINED CULTURES IN GUINEA PIGS

Passage No.	1	2	3	4	5
Inoculum (ml)	Culture mash 1 0	Exudate 0 08	Exudate 0 05	Exudate 0.05	Exudate 0.05
Trial 1	+P +P + +	++ +++P +++P ++++	+ + +P ++P	± +P +++P +++	+ ++ +++ +++
Trial 2	+ + +P ++P	+P +P + +	++P +++P +++ ++++	± + + +++P	+ + ++ ++
Trial 3	+P ++P ++ +++	+ + +P +++P	+++ +++ +++ +++P	+++ +++ +++P +++P	+ + + ++++
Control	+P ++ ++++ ++++	+ ++++P	++++* ++++* ++++ N S †	+P ++++	++++ ++++

Key ±, small nodular lesion, no exudate; +, less than 1 in localized abscess, ++, localized abscess 1 in or more in diameter; +++, localized abscess with extension, not fatal in 7 days; ++++, spreading infection fatal in 7 days or less; P, used for passage
The control inoculum was prepared from mixed culture of unpurified exudate
* Animals died before exudate could be aspirated.
† Nonspecific death in 1 day—no evidence of infection
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not capable of infecting guinea pigs under the conditions of these experiments (TABLE 2, Macdonald *et al* , 1956). It is significant that the process of deleting nonessential organisms permitted identification of one minimum combination, but not necessarily the only combination that would be capable of producing typical infections. In this respect it is worth noting that others have produced mixed anaerobic infections with different mixtures arrived at more or less arbitrarily (for example, D. T. Smith, 1930; Proske and Sayers, 1934; Altemeier, 1942, Ryff and Lee, 1946). The conclusion seems justified that these infections are bacteriologically nonspecific, but probably specific in the

* One strain of the original 17 was not used in these studies, as earlier experiments demonstrated it to be unnecessary.

that the growth factor was some analogue of vitamin K. A number of compounds were assayed for activity. The results are shown in TABLES 5 and 6.

The simplest compound satisfying the organisms' requirements appears to be a naphthalene structure with either a hydroxyl, an oxygen, or a carboxyl group in the alpha position. A methyl group or an amino group is not effective. Compounds having the required group in the beta position are not active. Various chemical groups in the 2, 3, or 4 position appear not to interfere with the activity Phthiocol was found active, in contrast to Lev's findings. Substitutions in other than the 2, 3, or 4 position appeared to render the structure inactive. Compounds with appropriate side chains but built on benzene, anthracene, or more complex ring structures were all found to be inactive.

It thus appears that the occurrence on mucous membranes of certain strains of *B melaninogenicus* depends on the elaboration by other organisms of a growth factor related to the K vitamins. *B. melaninogenicus*, in turn, has the potential of participating in the production of mixed anaerobic infections. It would

TABLE 4
SUBSTITUTION OF 2-METHYL-1,4-NAPHTHOQUINONE FOR *B. MELANINOGENICUS* GROWTH FACTOR

Medium	Optical density at 690 mμ after 2 days' incubation
Base (trypticase soy broth, and hemin, 5 μg /ml)	0 11
Base + 5% filtrate*	0 43
Base + menadione (1 0 μg /ml)	0 86
Base + menadione (0 5 μg./ml)	0 84
Base + menadione (0 1 μg /ml.)	0 24
Base + menadione (0 01 μg /ml)	0 09

* One-day culture of *Staphylococcus aureus* in peptone water, glass-filtered free of cells.

therefore be expected that any interruption in normal hygiene permitting a proliferation of vitamin K–producing microorganisms might also result in increased proliferation of *B melaninogenicus*. This would be particularly true in the presence of inflammatory reactions of the gingivae, where minute hemorrhages would provide a second absolute requirement of *B. melaninogenicus*, namely, heme.

In view of the dependence of *B. melaninogenicus* on the diphtheroid as a source of growth factor, it was of interest to determine whether the diphtheroid could be replaced in the pathogenic quartet by growth factor. Wheel-plate combinations of 3 organisms (the required organisms less the diphtheroid) were prepared on a blood-agar medium containing 0.5 μg /ml. of menadione. The resultant mixed growth was suspended in trypticase soy broth, 1 ml. of broth being used for each wheel-plate mixture. The suspension was given in 1-ml. inoculations to 15 guinea pigs divided into 3 groups. Group 1 was injected daily for 7 days with 0.1 ml. of water containing 5 μg. menadione bisulfite in the area inoculated with the wheel-plate mixture (the groin). Group 2 received similar inoculations with a solution containing 50 μg. of menadione bisulfite. Group 3 received 0 1-ml. inoculations of saline daily.

tivity. Autoclaving (121° C. for 15 min) resulted in a 95 per cent loss. Drying under vacuum at 20° C did not impair activity, nor did ethylene oxide or ultraviolet light. Ether extracts of the filtrate acidified with HCl to *p*H 1 5 were active. Evaporation gave a gummy residue soluble in NaOH or alcohol, and this redissolved residue retained the activity. Extracts of alkaline preparations of *p*H 11.5 were not active. Neither DPN nor DPNH could substitute for the growth factor The factor was absorbable onto charcoal and both anion and cation exchange resins (Dowex 21K and Dowex 50WX).

Sources of the factor were filtrates of a variety of organisms including strains of *Staphylococcus*, *Sarcina*, anaerobic streptococci, and anaerobic diphtheroids. *Treponema microdentium*, *Fusobacterium*, some diphtheroids, and some streptococci, both anaerobes and facultatives, failed to produce the factor. It was demonstrated to be present in whole saliva of man, maximum growth being

TABLE 3
ASSAY OF GROWTH FACTOR FOR *B MELANINOGENICUS*

Medium	Optical density at 690 mμ after 8 days' incubation
Base (thioglycolate broth)	0 0
Base + 0 1 ml heme*	0 0
Base + 0 1 ml heme + 20% filtrate†	0 44
Base + 0 1 ml heme + 5% filtrate	0 70
Base + 0 1 ml heme + 1% filtrate	0 85
Base + 0 1 ml heme + 0 1% filtrate	0 15
Base + 0 1 ml heme + 0.01% filtrate	0 03
Base + 5% filtrate	0 09
Base + 1% filtrate	0 01

* Prepared from horse blood cells washed three times and suspended in distilled water In later experiments purified hemin was used

† One-day culture of *Staphylococcus aureus* in peptone water, glass-filtered free of cells

supported by 20 per cent of ethylene oxide-sterilized saliva Cannulated parotid or submaxillary secretions were inactive

A number of strains of *B. melaninogenicus* from the oral cavity of man were tested for their dependence on the growth factor and on heme. Almost all required heme, but only certain strains required the growth factor. Those requiring the growth factor failed to ferment any of nine different carbohydrates, the independent strains were fermentative All strains, however, produced indole and hydrogen sulfide, and all hydrolized gelatin.

While these investigations were under way, two abstracts were published by Lev (1958 and 1959) reporting the requirement of a rumen strain of a black pigment-producing anaerobe for menadione (2-methyl-1,4-naphthoquinone), a synthetic vitamin K Several analogues were also reported to be active, and the activity was inhibited by one analogue, phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone).

Substitution for the growth factor with menadione demonstrated that this compound was able to replace the unidentified component of the filtrate (TABLE 4). The properties as described above were compatible with the hypothesis

the first passage developed infections. In the second passage 1 animal from the 50-μg./day menadione group and 2 animals from the saline group developed infections. No infections developed in the third passage. Cultures of exudate

TABLE 6

COMPOUNDS TESTED WHICH DID NOT REPLACE THE GROWTH FACTOR FOR *B. MELANINOGENICUS*

Benzoquinone	Hydroquinone	Pyrogallic acid	Phthalic acid	γ-Phenyl-N-butyric acid
Naphthalene	1-Amino naphthalene	1-Methyl naphthalene	β-Naphthol	
2,3-Naphthalenediol	Coumarin	1-Naphthol 3,6-disulfonic acid		
2-Methyl anthraquinone	Dicumarol			

indicated that *B. melaninogenicus* failed to survive or was present in very small numbers in all of the exudates.

The findings show that it is possible to produce what appear to be typical infections with a combination of 3 organisms rather than 4. However, since *B. melaninogenicus* did not survive in significant numbers, it seems likely that the dosages of menadione diffused away from the lesions too quickly to be

Where abscesses developed, they were aspirated and cultured aerobically and anaerobically. Exudate from two animals in each group was passed in 0.1-ml. dosage to 10 more animals (5 for each exudate). Animals in this

TABLE 5
COMPOUNDS REPLACING THE GROWTH FACTOR REQUIRED FOR *B. MELANINOGENICUS*

α Naphthol	1,3-Naphthalenediol	1,4-Naphthalenediol	Vitamin K_5
1,2-Naphthoquinone	1,4-Naphthoquinone	Menadione	Phthiocol
2,3 Dichloro-1,4-naphthoquinone	Vitamin K_1	Menadione bisulfite	α Tetralone
1,2-Naphthoquinone, 4-sulfonate	1 Naphthylacetate	α-Naphthoic acid	1,5-Naphthalenediol (active at 50 μg per ml. or higher)

* R = Phytyl group.

second passage received the same daily inoculations of saline or menadione as had the first-passage animals from which exudate was derived for inoculations. In 2 instances sufficient exudate was obtained from second-passage animals to pass to an additional group of 5 guinea pigs. Wherever exudate was obtained, it was cultured aerobically and anaerobically.

The results are shown in TABLE 7. It may be seen that all 15 guinea pigs of

among the pathogenic quartet known to be capable of producing typical infection.

A second set of tests was directed at determining the occurrence of active hyaluronidase in guinea pig exudates induced with the pathogenic quartet. Ampules of exudate from 2 animals infected with 4 organisms were removed from the Dry Ice chest. The exudates were lysed in a Raytheon Sonicator at 9000 cycles for 30 min. The lysate was centrifuged at 4600 rpm for 20 min., and the supernatant was assayed for hyaluronidase activity. The results are shown in TABLE 8, it may be seen that there was no evidence of hyaluronidase activity in these exudates.

These data suggest strongly that, although hyaluronidase production occurs in the complex mixed anaerobic infections of guinea pigs, it is not essential or even necessarily contributory to their pathogenesis. These findings obviously have no bearing on the possible role of hyaluronidase in periodontal disease from the standpoint of the enzyme's significance either in altering epithelial permeability or in permitting apical migration of the epithelial attachment.

TABLE 8

ASSAY FOR HYALURONIDASE ACTIVITY IN GUINEA PIG EXUDATE FROM INFECTION INITIATED WITH A COMBINATION OF 4 ORGANISMS

Reaction mixtures	Optical density at 350 mμ
Exudate lysate (0 1 ml) and buffer (0 9 ml)	0 0
Lysate and buffer + hyaluronate (200 μg), zero time	0 28
Lysate and buffer + hyaluronate (200 μg), 45 min at 37° C.	0 28
Testicular hyaluronidase (15 units) + hyaluronate (200 μg), zero time	0.32
Testicular hyaluronidase (15 units) + hyaluronate (200 μg), 45 min at 37° C	0.0

Evidence exists of a correlation between proteolytic activity of gingival microorganisms and the occurrence of periodontal disease. Berg *et al.* (1947) demonstrated faster hydrolysis of casein and greater productions of indole and H_2S by saliva of individuals with periodontal disease than by saliva of individuals with normal gingivae. Roth and Myers (1956) reported lysis of hide powder by 57 of 60 samples of gingival scrapings. Various altered collagens, in fact, are attacked by members of the oral microbiota (Lucas and Thonard, 1955, Schultz-Haudt and Scherp, 1955, Roth, 1957; Thonard and Scherp, 1957), but satisfactory evidence of degradation of native collagen has not been presented.

Preliminary experiments have been performed concerning the occurrence of proteolytic activity among the 4 organisms responsible for the guinea pig mixed infection. The production by broth cultures of indole or H_2S, end products of protein metabolism, is shown in TABLE 9. Indole production was tested in Difco thioglycolate broth except in the case of *B. melaninogenicus*, in which instance trypticase soy broth supplemented with hemin and menadione was used. H_2S production was determined in media supplemented with Difco peptone iron agar. It may be seen that 3 of the 4 strains produced indole and

effective in maintaining this organism. Hence the infection tended to disappear in the second passage.

A second bacterial metabolite of possible significance in periodontal disease and in the mixed infection under investigation is hyaluronidase. It has been shown that depolymerization of connective tissue ground substance of the gingivae by injection of testicular hyaluronidase in man results in an apical proliferation of the epithelium, and it has been suggested that this may be a mechanism contributing to deepening of the periodontal pocket (Aisenberg and Aisenberg, 1951). Schultz-Haudt *et al.* (1953) have presented evidence suggesting that hyaluronidase may increase the permeability of gingival epithelium, permitting ingress of hyaluronidase itself and other metabolites to the underlying connective tissue. In view of these observations, the demonstrated positive correlations between occurrence of gingivitis or periodontal disease and he levels of hyaluronidase in saliva, as well as the production of hyaluronidase

TABLE 7

GUINEA PIG INFECTIONS PRODUCED BY INOCULATION OF A COMBINATION OF 3 ORGANISMS, WITH AND WITHOUT SUBSEQUENT MENADIONE INOCULATIONS

	Menadione (5 μg /day)	Menadione (50 μg /day)	Saline (daily)
First passage (1 ml culture mash)	+, ++, ++, +++, +++	++, ++, +++, +++, ++++	++, ++, ++, ++, +++
Second passage (0 1 ml exudate)	0, ±, ±, ±, ± 0, 0, 0, 0, ±	±, ±, ±, ±, ++ 0, 0, ±, ±, ±	0, ± ± ± + 0, 0, 0, 0, +
Third passage (0 1 ml exudate)		0, ±, ±, ±, ±	±, ±, ±, ±, ±

Key ±, small nodular lesion or diffuse inflammation, little or no exudate, +, less than 1-inch localized abscess, ++, localized abscess 1 inch or more in diameter, +++, abscess with extension, not fatal in 7 days, ++++, spreading infection fatal in 7 days or less

by oral organisms, assume added significance (Schultz-Haudt and Scherp, 1955, Mahler and Lisanti, 1952)

Tests were made for production of hyaluronidase by all 16 strains persisting in the guinea pig mixed infection. Two methods were employed The first was an adaptation of the colorimetric method of Greif (1952), dependent on the stoichiometric combination of Bromsulphalein with a hyaluronic acid-plasma protein complex and subsequent redissolving of the complex in alkaline solution to release the indicator. Optical density was determined at a wave length of 575 mμ. Equally satisfactory and simpler for qualitative studies was a turbidimetric assay. Hyaluronic acid (200 μg) in 0 5 ml. of acetate buffer (*p*H 6 0) was used as substrate. To this was added 0 5 ml. of the culture or culture supernatant, and the mixture was incubated at 37° C. for one hour. Optical density was determined at 350 mμ and compared to suitable controls with known concentrations of hyaluronate

It was demonstrated by these methods that 5 of the 16 strains produced hyaluronidase Three of these were anaerobic streptococci, one was an anaerobic diphtheroid, and one was a *Bacteroides* species. None of the 5 was

They fall far short of explaining the pathogenesis of this mixed anaerobic infection. Nevertheless, they appear to throw some light on the subject. They point to a significant role for *B. melaninogenicus*. This organism is present regularly in the oral cavity; it is our impression that its numbers are strikingly increased in periodontal disease. We lack satisfactory quantitative data to support this view. The organism appears to be essential to the production of the guinea pig infection under study for, when it has been omitted from recombination mixtures, no lesions have occurred. The organism does not depend on simple carbohydrates as an energy source; on the other hand, it is characterized by a capacity to hydrolize protein and it is possible that this proteolytic activity may be significant in the production of both the guinea pig infection and human periodontal disease. The demonstration of strong proteolytic activity against gelatin by lysates of guinea pig exudate supports this view.

The studies demonstrate the existence of a specific nutritional requirement of *B. melaninogenicus* that is met by the synthetic capacities of various members of the oral microbiota. This synthesis of vitamin K-like substances has been demonstrated to occur in the oral cavity and appears to be a mechanism controlling the occurrence of *B. melaninogenicus* on oral mucous membranes.

It has been shown also that production of hyaluronidase is accomplished by various organisms persisting in the complex guinea pig mixed infection. At the same time, comparable infection can be produced with less complex mixtures in which none of the components elaborates hyaluronidase. Evidently, therefore, although hyaluronidase may have a role in periodontal disease, it is not essential to the pathogenesis or maintenance of infection.

In the case of the guinea pig infection no significant clues concerning the biochemical role of 2 of the 4 organisms have been disclosed. It is hoped that further studies may throw light on these problems and help to elucidate the mechanisms involved in this unusually complex type of host-parasite relationship.

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one produced H_2S. It is of some interest that 4 additional strains in the constellation of 16 produced indole. One additional strain produced H_2S.

The occurrence in the 4 organisms of a gelatinase was sought. Each organism was grown in thiogel medium (B.B.L.). The cultures were incubated anaerobically at 37° C. for 5 days, refrigerated for 2 hours and examined for liquefaction of the gelatin. Only one strain, *B. melaninogenicus*, liquefied gelatin under these conditions.

The presence of gelatinase was tested for in guinea pig exudate containing the 4 organisms. Exudate was lysed by ultrasonic vibration at 9000 cycles for 30 min. The lysate was centrifuged, and the supernatant decanted through

TABLE 9
THE PRODUCTION OF INDOLE AND H_2S BY THE 4 ESSENTIAL ORGANISMS IN A GUINEA PIG MIXED INFECTION

	Indole	H_2S
B melaninogenicus	+	+
Bacteroides species	+	0
Motile Gram-negative anaerobe	+	0
Facultative diphtheroid	0	0

TABLE 10
GELATINASE ACTIVITY OF GUINEA PIG EXUDATE FROM INFECTION INITIATED BY A COMBINATION OF 4 ORGANISMS

	Flow time (sec)	
	0 hours	30 min
Exudate lysate (2 ml) + 7% gelatin (2 ml)	14 0	9 4
Lysate + gelatin, inactivated at 100° C , 10 min	29 3	29 3
C histolyticum lysate (2 ml) + 7% gelatin (2 ml)	16 6	8 3
C. histolyticum lysate + gelatin inactivated at 100° C., 10 min	20 8	21 2

Zero-hour flow time was shorter for active preparation than for inactivated preparations, presumably because hydrolysis occurred in the short time involved in recording the readings.

glass wool. An aliquot was inactivated by heating to 100° C. for 10 min. Seven per cent gelatin in 0.05 M trihydroxymethylaminomethane buffer (*p*H 7.2) was used as substrate. Two milliliters of the test substance was added to 2 ml. of substrate and the mixtures were incubated at 37° C. Viscosity was measured as flow time through a capillary pipette at 15-min. intervals for 45 min. A culture of *Clostridium histolyticum* was treated in the same way as the exudate and was used as a positive control. The results are shown in TABLE 10. It may be seen that the exudate decreased the viscosity of the gelatin at approximately the same rate as did the *C. histolyticum* preparation. It thus appears that the exudate from guinea pigs infected with the 4 organisms contains an active protease capable of hydrolyzing gelatin. It appears also that *B. melaninogenicus* is the probable source of this protease.

These studies are obviously incomplete and the findings are fragmentary.

OXIDATIVE ENZYMES OF GINGIVA*

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With reference to Lisanti's paper and introductory remarks elsewhere in this monograph, our meeting in 1953, mutual interests, and respective work in progress resulted in our decision that the best approach to understanding the oral cavity in health and disease lay in establishing a sound foundation for the associated problems by means of direct enzyme, tissue, and cell-metabolism studies. To exemplify this: were one to consider the elucidation of the problem of periodontal disease and, specifically, of the human gingiva, its resolution could be achieved through the comprehensive and simultaneous evaluation of a broad spectrum of enzymes, coenzymes, cofactors, and intermediates within the gingival tissue complex. Since such information did not exist, and with these objectives in mind, study of the gingiva was initiated in 1953 to determine base-line levels of activity for several oxidation-reduction enzymes with the view of ultimately establishing patterns of metabolic activity that would enhance our understanding of the gingiva.

Phase 1

Experimental

In the first phase of this work the rabbit was chosen as the tissue donor, since sufficiently large samples of gingiva and other tissue complex masses could be harvested for study. Liver, brain cortex, submaxillary gland, tongue mucosa, tooth pulp, retina, choroid, and the crystalline lens were selected for study, in addition to the gingiva, to establish a comparative evaluation for a large representative group of organs. Six oxidation-reduction enzymes were investigated, each enzyme occupying a key position within the anaerobic or aerobic glucose metabolic pathways. The enzyme activities detected were expressed as the specific activities per milligram of protein to reflect the concentration of total enzyme present in a unit amount of tissue The results obtained have appeared elsewhere as a series of papers [1-5] The data for succinic dehydrogenase (SD), DPNH cytochrome *c* reductase (DPNHCR), cytochrome *c* oxidase (Cyt Ox), catalase (C), glucose-6-phosphate dehydrogenase (G-6-PD), and pyruvic reductase (PR), obtained from a group of 17 New Zealand white rabbits and, for catalase (C), from a group of 11 New Zealand white rabbits, have been re-expressed and are summarized in TABLE 1.

A mean value of 1 0 has been assigned to the gingiva enzyme activities. Relative mean homogenate enzyme activity values for the other tissues studied are presented. Data for the crystalline lens have been omitted. It can be seen that rabbit gingiva SD, DPNHCR, and Cyt Ox activities can be classed

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prior to placement in the reaction tubes. A modified method of Nachlas *et al.*[7] was employed for the disclosure of endogenous reducing enzyme and SD activities. The concentrations of reactants, their order of addition, and the various reaction mixtures used are given in TABLE 2. Five tissue sections were placed in each reaction tube. The tubes were inserted in a rack adapted to an Eberbach variable-speed shaker and incubated for 30 min. in a constant-temperature bath maintained at 35° C. The rate of shaking was 120 oscillations/min. The principle of shaking was introduced to assure the best possible conditions for the diffusion of reactants into the tissue sections.

At the end of the reaction time the sections were rinsed with distilled H_2O for 1 min. and placed in 10 per cent formalin for 15 min. to stop further reaction. The sections were then rinsed in distilled water at least 4 times, placed on slides, drained, covered with a drop of neutral glycerine, dehydrated in a vacuum desiccator, and covered bubblefree.

Spectrophotometric enzyme analyses. All tissue samples weighed 30 to 50 mg.

TABLE 2

CONCENTRATION OF REACTANTS AND THEIR ORDER OF ADDITION FOR THE HISTOCHEMICAL LOCALIZATION OF ENDOGENOUS REDUCING ENZYME AND SUCCINIC DEHYDROGENASE ACTIVITIES

Reaction mixture	1 0 1 M KH_2PO_4-Na_2HPO_4 buffer *p*H 7 4 (ml)	2 5 × 10^{-3} M NaCN in phosphate buffer (ml)	3 0 1% Nitro BT in phosphate buffer (ml)	4 0 5 M Sodium succinate in phosphate buffer (ml)
1	2 0	0 0	1.0	0.0
2	1 0	1 0	1 0	0 0
3	1 5	0 0	1 0	0 5
4	0.5	1 0	1 0	0 5
5	1 5	1 0	0 0	0 5

by wet weight. The homogenates were prepared in Ten Broeck glass homogenizers. The tissues were suspended in ice-cold 0.05 M KH_2PO_4-Na_2HPO_4 buffer (*p*H 7.4) to yield a 1:100 dilution, wt /vol , and stored in a cracked-ice bath

SD, DPNHCR, PR, G-6-PD, and Cyt Ox activities were quantitatively measured with the Beckman Model DU quartz spectrophotometer. The concentrations and order of addition of reactants for SD, DPNHCR, and Cyt Ox were in accord with Eichel and Swanson[1-3] except for the following modifications: 0.3 ml. of tissue homogenate was employed in each assay; 1.0 mg. of beef heart cytochrome *c* by dry weight was added to each reaction cuvette. G-6-PD was assayed in accord with Eichel,[4] and PR in accord with Eichel *et al.*[5] Modifications included here were as follows: for G-6-PD assay, TPN was added to the tissue prior to homogenization with the PO_4 buffer to yield 1.0 mg. TPN/1.0 ml. of homogenate; 0 3 ml. of the homogenate was used in the assay for G-6-PD, and 0 05 ml. of homogenate in the PR assay.

SD and DPNHCR activities were determined by following the conversion of ferricytochrome *c* to its reduced form at 550 mμ. Cyt Ox activity was determined by following the conversion of ferrocytochrome *c* to its oxidized form

among those tissues having low enzyme activities. For C, the gingiva can be classed among those tissues having intermediate activity; only the liver possessed fourfold greater activity than the gingiva. For G-6-PD the gingiva can be classed among those tissues having intermediate activities. Only the retina contained 4.5 times greater activity than the gingiva. For PR, the retina yielded activity values seven times higher, the choroid approximately two times higher, and the liver 1.5 times higher than that of the gingiva. Recently the second phase of this investigation, inquiring into the status of these oxidative enzymes in human gingiva, has been undertaken.

TABLE 1

NEW ZEALAND WHITE RABBIT TISSUE HOMOGENATE ENZYME ACTIVITY MEAN RELATIVE VALUES COMPARED TO GINGIVA

(A Mean Value of 1.0 Has Been Assigned to the Gingival Enzyme Activities)

Tissue	Succinic dehydrogenase*	DPNH Cytochrome c reductase*	Cytochrome c oxidase*	Catalase†	Glucose-6-PO4 dehydrogenase‡	Pyruvic reductase§
Gingiva	1.0	1.0	1.0	1.0	1.0	1.0
Submaxillary gland	12.0	20.0	17.0	0.7	0.1	0.9
Tongue mucosa	5.5	5.0	6.5	—	1.3	1.0
Tooth pulp	2.4	2.5	1.3	1.0	0.3	0.8
Liver	7.2	25.0	11.0	4.2	0.4	1.5
*Brain cortex	9.0	—	—	0.3	0.3	1.0
Retina	1.5**	1.4**	—	0.3	4.5	7.0
Choroid	1.3**	1.7**	—	1.4	1.1	1.8

* Calculated from Eichel and Swanson,[1,2] experiment No. 2, Figures 4, 6, and 8, and Table II (17 rabbits studied).
** Calculated from Eichel and Swanson,[3] Tables II and III (6 of 17 rabbits studied).
† Calculated from Eichel and Swanson,[3] Table I (11 rabbits studied)
‡ Calculated from Eichel,[4] Figure 4 and Table III (15 of 17 rabbits studied)
§ Calculated from Eichel *et al.*,[5] Figure 3 and Table I (12 of 17 rabbits studied).

PHASE 2

Methods

Handling of gingiva. Only human gingiva that had been preoperatively treated prior to gingivectomy was studied. The gingiva was sampled by vertically cutting in half one interdental papilla with its marginal buccal or lingual extensions. One half was taken for preparation of frozen sections; the other half was taken for homogenate enzyme assays. A third sample of gingiva adjacent to that taken for frozen sectioning, was removed to prepare a separate homogenate for G-6-PD assay, that is, the addition of TPN* to the homogenate during its preparation for the protection of the labile G-6-PD.[6]

Histochemistry. All tissue samples weighed 30 to 50 mg. by wet weight. Several frozen sections 25 μ in thickness were prepared and immediately placed in 10 per cent formalin for subsequent eosin-hematoxylin staining and histological examination. From the remaining gingiva sample 30-μ-thick sections were prepared and immediately placed in ice-cold 0.1 *M* PO_4 buffer (*p*H 7.4)

* Oxidized triphosphopyridine nucleotide

dark purple or blue-purple; see above) and also because the intensity of colored material in the superficial layers is probably of a lower order of magnitude than that of the basal cell layer.

Keeping these factors in mind, the subjective evaluation of activity is presented in FIGURE 2. The individual values are represented by ovals, the mean values by bar heights. It may be seen that the activity increased in the superficial layers of the epithelium in the presence of succinate (diagonal line bar, endogenous reducing plus SD activity, compared to horizontal line bar, the endogenous reducing activity). The SD activity appears to be smaller in magnitude that that of the endogenous activity. The addition of cyanide to the

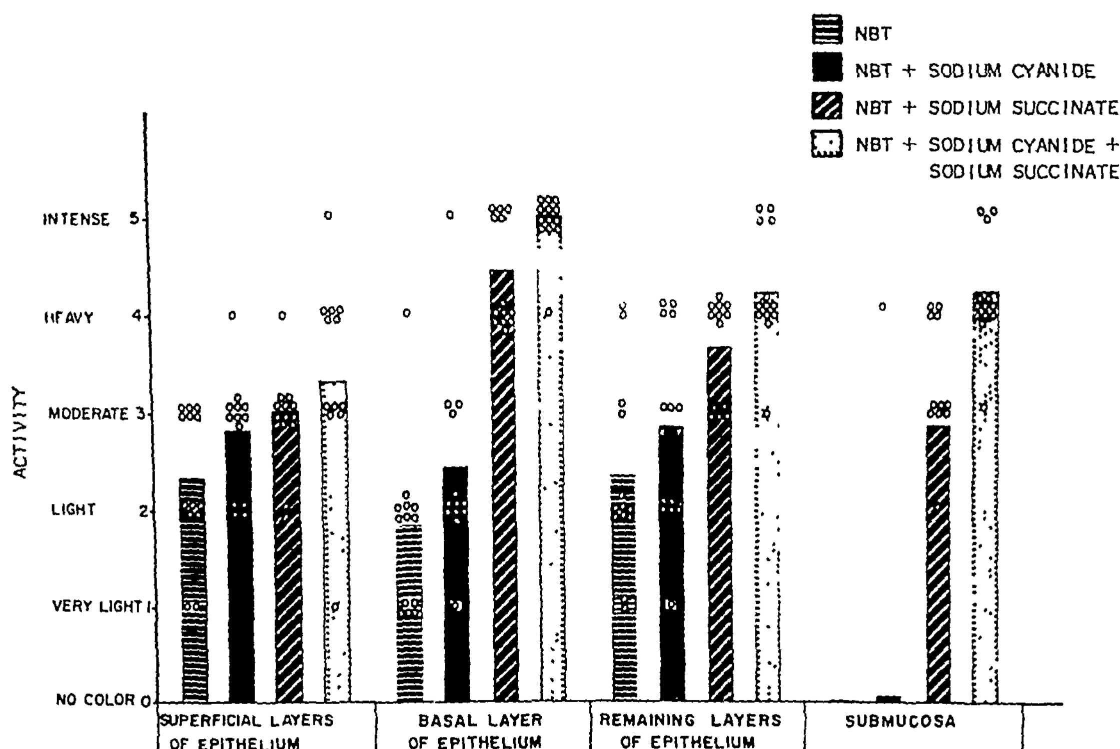


FIGURE 2 Evaluation of histochemically disclosed endogenous reducing and SD enzyme activities in various areas of human gingival tissue sections

system without succinate (solid bar) and to the system with succinate (stippled bar) demonstrates a slight increase in endogenous and SD activities, respectively.

For the basal cell layer of the epithelium, the presence of succinate (diagonal-line bar) pronouncedly enhanced purple formazan formation when compared to the substrateless system (horizontal-line bar). Again, the addition of cyanide enhanced activity. The activities present in the remaining layers of the epithelium fall somewhere between the activities seen in the superficial and basal cell layers. The pattern evolved parallels the conventional understanding of the viability of mucosal epithelial tissue cells and their probable life cycle.

In the submucosa the pattern changed. Here endogenous reducing enzyme activity could not be visualized in the absence or presence of sodium cyanide. The addition of succinate, however, showed definite SD activity (diagonal-line

at 550 mμ. PR activity was determined at 340 mμ by following the conversion of reduced diphosphopyridine nucleotide (DPNH) to its oxidized form. G-6-PD activity was determined by following the conversion of TPN to its reduced form at 340 mμ. All reactions were performed in duplicate.

For the calculation of reaction rates each 0 001 density change was defined as 1.0 density unit The specific enzyme activities were determined on the basis of the density unit change per 300 sec. per unit amount of tissue homogenate.

Results

Qualitative Enzyme Analyses

The histochemical disclosure of endogenous reducing enzyme and SD activities in human gingiva, under the controlled conditions employed, are given in typical photomicrographs in the FRONTISPIECE (FIGURES 1*a* and *b*, *upper*).

Endogenous reducing enzyme activity in the absence of added substrate and cyanide was made evident by the light pink-purple color in the prickle cell and most superficial layers of the epithelium. No color could be detected in the submucosa. In the presence of cyanide the intensity of the color in the epithelial tissue increased, suggesting the possible participation of oxidizing systems in the epithelium. No color could be detected in the submucosa except in one case.

Evidence for the presence of increased activity was obtained by the greater color intensity observed when sections were incubated in the presence of succinate (FIGURE 1*a*) The basal cell layer and epithelial pegs showed the presence of purple formazan. The prickle cells above were lighter purple, and the color showed a gradient change to pinkish purple as the superficial layers were approached The submucosa yielded definite violet to purple color which, under high-power examination, was essentially associated with blood vessels and areas of cellular infiltration. The purple formazan deposits are taken as evidence for SD activity. The addition of cyanide to the succinate system definitely intensified reaction (FIGURE 1*b*). Blue color became visible at times in the basal cell layer and epithelial pegs. The intensification of color production in the presence of cyanide substantiates the conclusion that oxidizing systems interfere to some extent with the demonstration of reducing enzyme activity when employing the tetrazolium salt nitro BT under controlled conditions.

A total of 13 human gingiva biopsy samples was sectioned, processed, and studied as indicated above. Color intensity, as a measure of activity, was graded into 6 groups, ranging from (1) no color, through (2) very light, (3) light, (4) moderate, (5) heavy, to (6) intense color (FIGURE 2). The tissue was divided into 4 areas for evaluation. (1) superficial layers of epithelium, (2) basal layer of epithelium, (3) remaining layers of epithelium, and (4) submucosa.

Although the relative color intensity for a given area is a real impression of the degree of activity for the area, it should be noted, for example, that a designation of intense activity for the superficial layers of epithelium can not be equated to intense activity of the basal cell layer. This is true because the colors seen in the 2 layers are distinctly different (pinkish-purple in contrast to

course of reaction for a 30-min. period in the presence and absence of sodium cyanide. The very low rate of activity observed in the presence of sodium cyanide was undoubtedly caused by the method adopted for the reaction assay.*

G-6-PD could be demonstrated only in homogenates prepared with added TPN. Repeated attempts to identify G-6-PD activity when TPN was not added to the homogenate but added instead to the reaction mixture in the

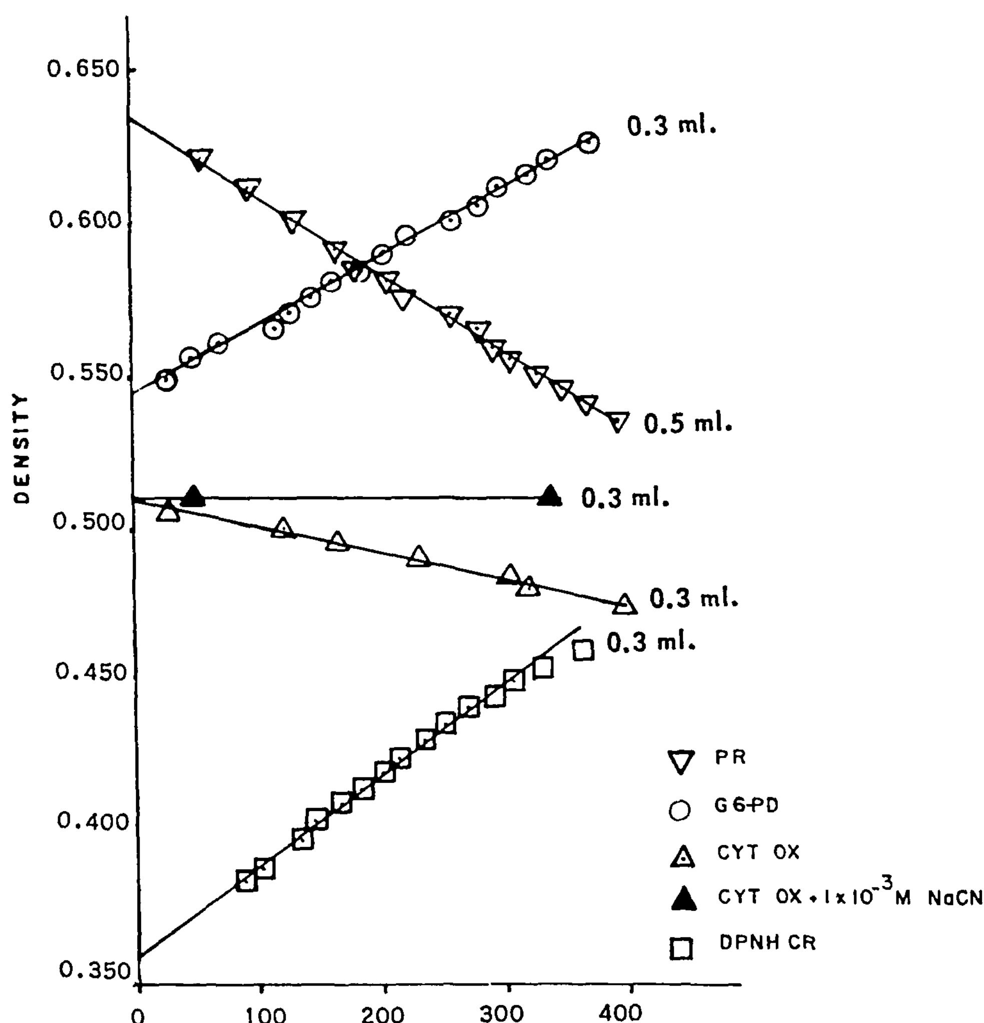


FIGURE 3. Human gingival homogenate PR, G-6-PD, and Cyt Ox in the presence and absence of sodium cyanide, and DPNHCR reaction rates.

cuvette met with failure. The need for the direct addition of TPN to the homogenate for the estimation of G-6-PD demonstrates the probable labile nature of G-6-PD in broken cell human gingiva preparations. This observa-

* The complete reaction mixtures were pipetted into test tubes placed in a rack adapted to a shaker. The rack containing the tubes was immersed in a constant temperature bath maintained at 35° C. The tubes were shaken at the rate of 120 oscillations per minute. At 5-min intervals the content of the tubes was decanted into reaction cuvettes and read directly in the Beckman spectrophotometer. After reading, the reaction mixtures were poured back into the test tubes and incubated. The repeated pouring of the reaction mixtures from test tubes to cuvettes and from cuvettes to test tubes probably diluted the volatile cyanide, resulting in the slight reaction detected.

bar), which increased in the presence of cyanide (stippled bar). Under the controlled conditions employed here, it is obvious that the presence of cyanide influences the degree of formazan formation, probably by means of cyanide inhibition of Cyt Ox. One of 2 mechanisms of action, or both, may be occurring. First, Cyt Ox may reoxidize some of the formazan back to the ditetrazole, or a typical succinoxidase system may compete with the succinic dehydrogenase-nitro BT system for hydrogen ions and electrons.

SD activity for 3 gingival samples was quantitated by spectrophotometric analysis Since the number of tissue samples studied was too small, the specific results are not included here However, the activity was comparable to that of Cyt Ox discussed below.

Several of the above observations are at variance with some of the findings and conclusions of Nachlas *et al.*[7] First, these investigators stated that no red color (monoformazan) was seen in any of their uncounterstained tissue sections, and they concluded that monoreduction of the ditetrazole was not a significant phenomenon with nitro BT They also noted, however, that when their sections were dehydrated with alcohol and cleared with xylol, pink coloration appeared in their alcohol solutions. Alcohol dehydration and xylol clearing was not employed in our procedure The end result was the visualization of pinkish-purple, purple, as well as blue color-staining reactions, even when steps were taken to remove possible traces of monoformazan as a contaminant present in the nitro BT reagent. Blue color-staining reaction was occasionally seen in gingivae under the conditions employed here. Blue color could always be seen when SD activity was disclosed in highly active tissues, for example, striated tongue muscle.

Second, Nachlas *et al.* stated that the use of buffered KCN showed no significant improvement in staining. Our results indicate that the presence of cyanide enhances reaction in our test system.

Third, Nachlas *et al.* indicated that no stain was evident in dog kidney sections after incubation for one hour in the absence of succinate. Under the conditions of assay outlined herein, staining occurred in gingival epithelium, indicating that some endogenous reducing activity can be disclosed in some reactive tissues.

Quantitative Enzyme Analyses

Cyt Ox (in the presence and absence of cyanide), G-6-PD, PR, and DPN-HCR reaction rates for a single human gingival biopsy sample are given in FIGURE 3. Each of the reactions yielded straight lines with respect to time when plotted on Cartesian coordinate paper. At first, the slow Cyt Ox activity was thought to be due to possible gradual settling of homogenate sediment rather than to actual reaction. Consequently, parallel reactions were studied in the presence of 1.0×10^{-3} *M* NaCN (final concentration in the reaction cuvette). No change in density could be detected within a 6-min. period, indicating that the apparent slow rate of activity found for Cyt Ox was the actual measure of a small concentration of enzyme. The presence of Cyt Ox activity was more dramatically demonstrated (FIGURE 4), employing a gingival tissue homogenate from another human subject, by following the

sons could readily be made between activity levels for the several enzymes. These data are summarized in FIGURES 5 and 6. In FIGURE 5 each circle represents the average value for duplicate enzyme assays obtained for each homogenate sample by volume size taken for study. The bar heights give the mean values for the respective enzymes. The mean values have been re-expressed in FIGURE 6 by relating the activities to a constant volume of homogenate. The bar heights give the values for Cyt Ox, G-6-PD, DPNHCR, and PR in

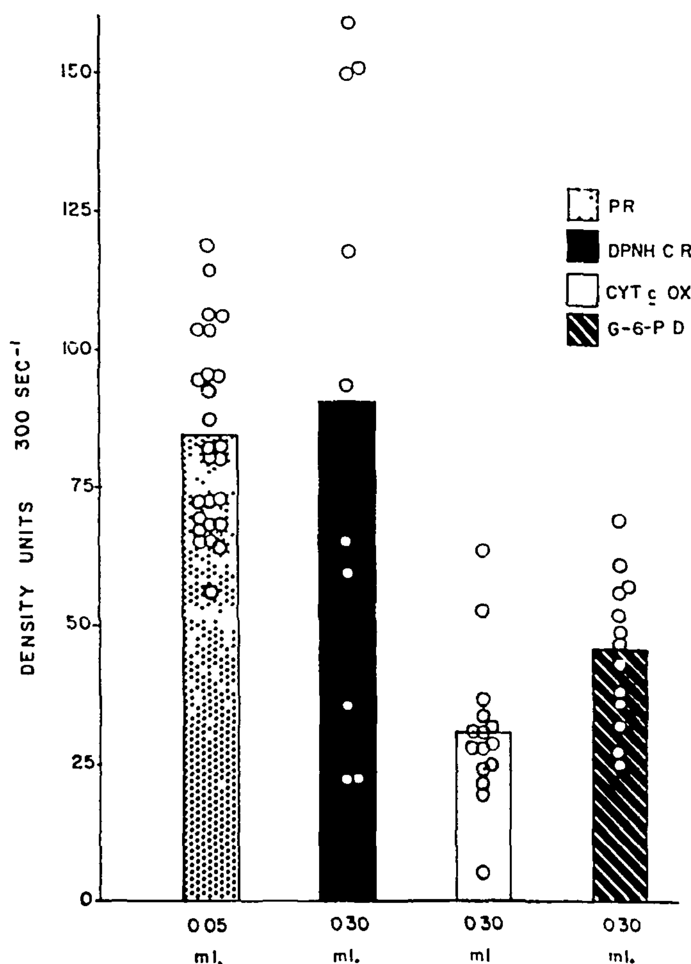


FIGURE 5 Mean and individual PR, DPNHCR, Cyt Ox, and G-6-PD activities of human gingival homogenates.

the ascending order of their activity potential. These human gingivae contain PR activity 17 times greater than the Cyt Ox activity, 11 times greater than the G-6-PD activity, and 6 times greater than the DPNHCR activity.

Several points of interest emerge here:

(1) If these human gingivae were truly pathological, then Cyt Ox as the terminal respiratory enzyme and thus the one of possibly greatest importance studied here was present in so low a concentration to indicate that aerobic activity would be limited, and anaerobic activity would predominate in this tissue complex. This possibility is fortified by the very high pyruvic reductase activity.

tion correlates with those previously made employing rabbit tissue homogenates.[6]

Twenty-six gingiva homogenate preparations from 9 human subject biopsy samples were studied for PR activity, 10 homogenates from 5 subjects for DPNHCR activity, 15 homogenates from 7 subjects for Cyt Ox activity, and 13 homogenates from 7 subjects for G-6-PD activity The reasons for the difference in numbers of homogenates and subjects studied for each enzyme were as follows: (1) PR activity was demonstrated readily in all homogenates even in the presence of TPN; (2) DPNHCR activity was studied only in biopsy

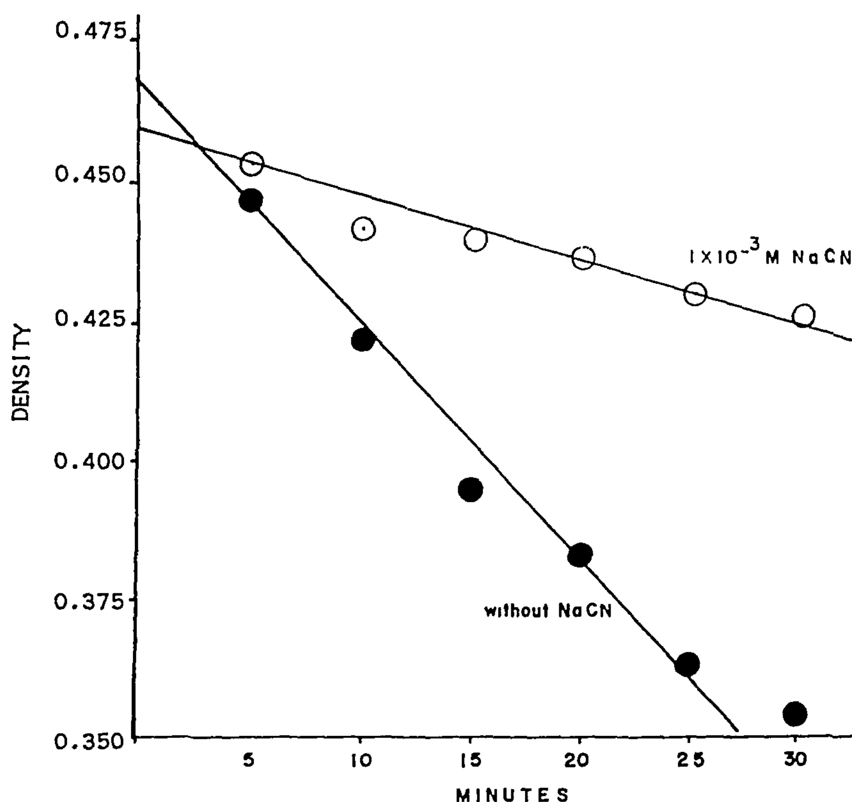


FIGURE 4 Inhibition of human gingival homogenate Cyt Ox activity by sodium cyanide

samples obtained from the last 5 subjects; (3) Cyt Ox activity was sufficiently doubtful in several of the first homogenates studied to result in the conclusion that activity was absent (these data were not included in the Cyt Ox results given in FIGURES 5 and 6); (4) several homogenates prepared for G-6-PD analysis were lost during preparation; and (5) DPNHCR and Cyt Ox could not be assayed in the TPN homogenates prepared for G-6-PD assay.*

The velocity constants for all reactions were analyzed in accord with zero-order reaction kinetics (the change in concentration of the substance being followed is directly proportional with respect to time). In this fashion compari-

* It is possible that the added TPN would have mediated the reduction of cytochrome *c* in the presence of endogenous substrate, TPN dehydrogenases, and TPN cytochrome *c* reductase present in the homogenates

Endogenous reducing and succinic dehydrogenase activities have been disclosed under controlled conditions, employing modification of the nitro BT method. Pink-purple, purple, and blue staining reactions have been observed. Cyanide has been used, and it intensified reaction, suggesting the participation of oxidizing systems. A graphic summary of the subjective evaluation of the various staining reactions for different areas of the gingiva has been given.

Several oxidative enzymes have been spectrophotometrically quantitated in homogenates of these human gingivae. Succinic dehydrogenase activity appears to be low. Quantitative activity data for several other enzymes has been included. Comparison of the activity levels has demonstrated that pyruvic reductase (lactic dehydrogenase) is 17 times greater, DPNH cytochrome *c* reductase is 3 times greater, and glucose-6-PO_4 dehydrogenase is 1.5 times greater than cytochrome *c* oxidase in human gingiva. The latter definitely establishes the limiting nature of the terminal respiratory enzyme in these human gingival samples.

Some of the implications of these findings and their significance has been discussed.

ACKNOWLEDGMENTS

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(2) If these gingivae were similar to the normal, however, then the small amount of Cyt Ox activity and high PR activity would reflect the type of enzyme pattern found in the crystalline lens, cortex, and nucleus of rabbits (unpublished experiments) and the metabolic pattern found in jejunum mucosa,[8] cartilage,[9] synovial membrane,[10] and skin epithelium.[11,12] The facts that jejunum mucosa and skin epithelium possess high glycolytic and low respiratory activities and that the gingiva PR and Cyt Ox activities parallel these metabolic patterns lends credence to the suggestion that the gingivae studied here more closely resembled the normal rather than the pathological.

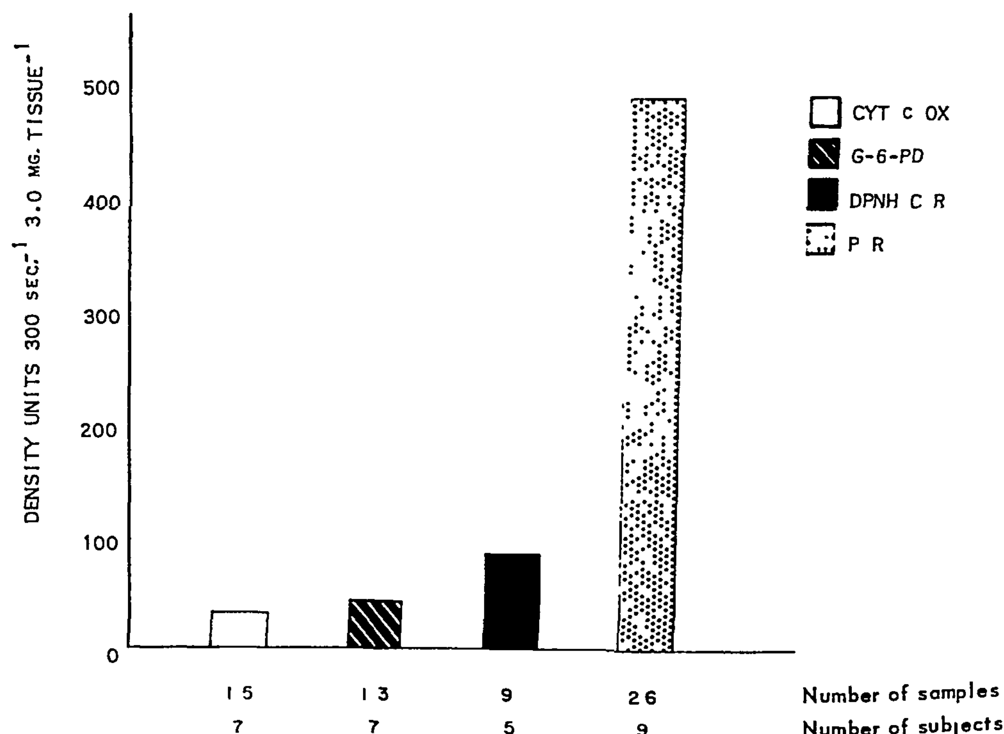


FIGURE 6 Comparative mean specific Cyt Ox, G-6-PD, DPNHCR, and PR activities of human gingival homogenates.

(3) The conclusion that Cyt Ox in these gingivae was limiting is strengthened by the observation that G-6-PD activity was 1 5 times greater and DPNHCR activity was 3 times greater than the Cyt Ox activity.

Continuing studies are in progress.

Summary and Conclusions

Succinic dehydrogenase, DPNH cytochrome *c* reductase, glucose-6-PO_4 dehydrogenase, cytochrome *c* oxidase, and catalase activities have been quantitated in rabbit gingiva, tongue mucosa, tooth pulp, submaxillary gland, retina, choroid, liver, and brain cortex homogenates The relative enzyme activities for each tissue homogenate have been compared, assigning a value of 1 0 to the gingival activities.

Human gingival biopsy samples, preoperatively treated prior to gingivectomy, have been studied.

(FIGURE 2). A fuchsinophilic band of transseptal fibers was also routinely noted in the noninjured interdental papilla, in association with the normally occurring gingival inflammation resulting from food impaction. Such changes



FIGURE 1 Mesiodistal section of human mandibular first molar Fuchsinophilic (dark staining) transseptal fiber bundles below ulceration [5] Allochrome stain; ×75.

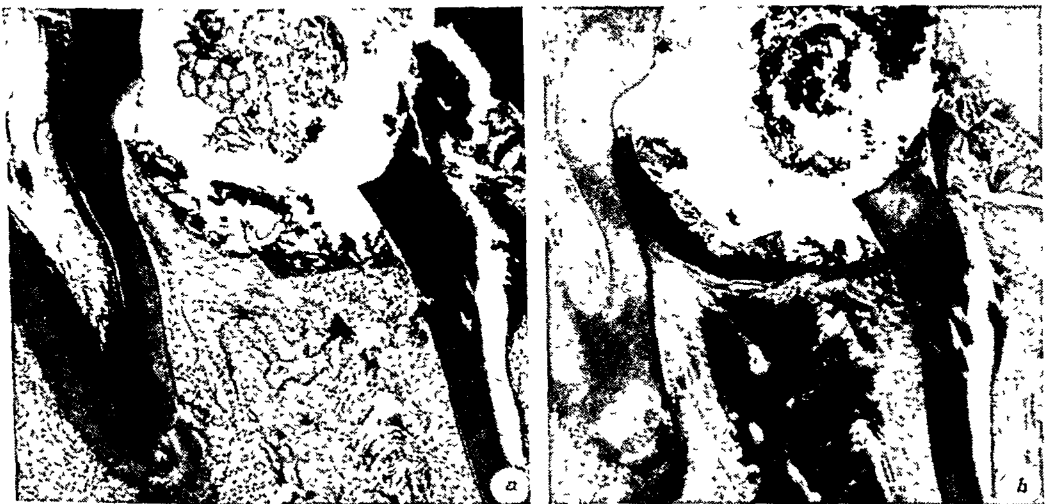


FIGURE 2 (*a*) Mesiodistal section of first and second molar of a rat 6 hours after gingival interproximal injury [6] (*b*) Serial section of specimen shown in *a* Fuchsinophilic (dark staining) transseptal fibers below injury PAS stain; ×60

denote an alteration in a very significant structure of the periodontal attachment apparatus at a level distant to the deepest penetration of the epithelial attachment that occurs prior to the morphologically visible destruction of the fibers. According to present biochemical concepts, these changes probably involve enzymatic mechanisms. The importance of the transseptal fiber bundles to the defense of the periodontium against injury has been pointed out

INTERACTION BETWEEN LOCAL AND SYSTEMIC FACTORS UPON THE GENESIS OF PERIODONTAL LESIONS*

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Periodontal disease has often been described as the result of an interaction of such factors as gingival irritation, occlusal trauma, and systemic imbalances. Since the exact role of each of these contributing agents can seldom be defined in a clinical situation,[1] procedures must be devised that will enable us to study the adaptation of the periodontal tissues to these insults acting individually and in combination

Accordingly, the present paper will discuss (1) the experimentally observed response of periodontal soft tissues to local and systemic insults, (2) the response of alveolar bone to systemic stressors, and (3) the response of the periodontium to simultaneously occurring, multiple insults. It also will attempt to correlate the experimental findings with clinical observations.

Response of Soft Tissues of the Periodontium to Local and Systemic Insults

Let us first consider the response of the periodontal tissues to gingival irritation. It is commonly accepted that such irritants as calculus and overhanging margins produce an ulceration with apical proliferation of the epithelial attachment. The inflammatory reaction also causes changes in the fiber attachment and ultimately produces resorption of the alveolar crest. According to this well-proved concept, a known irritation acts as the initiating factor In a great number of clinical cases, however, no such simple irritant can be visualized, and it is thus suggested that in some cases the exciting local agent may not be a purely mechanical injury. In accordance with a concept that recently has received new stimulus, bacteria and bacterially produced enzymes may initiate the pathological condition.[2-4] While no definitive data are available there is increasing evidence of a relation between enzymatic activity and the pathological changes observed in periodontal lesions An example (FIGURE 1), is an area of gingival and transseptal fibers below a chronic gingival ulceration in a human specimen. By the use of a modification of the periodic acid–Schiff stain it was observed that the transseptal fibers near the surface ulceration were much more fuchsinophilic than the more apically located fibers. Further identification of this fuchsinophilic material suggested that it was composed partially of hyaluronic acid and some mucoproteins.[5] Recent studies of the healing of experimentally induced gingival wounds in rats have revealed a marked lability of the ground substance in the transseptal bundles within twenty-four hours of injury. Here too a partially hyaluronidase-labile increased fuchsinophilia in the transseptal bundles below the injury appeared similar to the transseptal fiber change seen in the human specimens

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is similar to the alveolar bone changes in tumor-bearing animals. It is of further interest that intraoral roentgenograms taken prior to operation did not show any abnormal trabeculation. It is thus possible that many of the fine details of bone architecture, which reflect the constant state of metabolic flux



FIGURE 3 (*a*) Interradicular septum of first molar of control rat. H&E stain; ×60. (*b*) Interradicular septum of first molar of rat on protein-free diet for 6 weeks [27] Enlarged marrow cavity H&E stain, ×60 (*c*) Interradicular septum of first molar of rat bearing abdominal tumor [19] Enlarged marrow cavity H&E stain; ×65. (*d*) Interradicular septum of first mandibular molar from patient with carcinoma of the tongue Enlarged marrow cavity. H&E stain, ×10.

in this tissue, are obscured in roentgenograms and lost to the clinician observing the patient.

If the above hypothesis is valid, then, conversely, the subjection of alveolar bone to anabolic stimulation should also be observable. Recent experiments with such hormones as estrogens[22-24] and somatotropic hormone[25] have demonstrated such a growth of the endosteal bone at times as almost to obstruct the

by Goldman who, from histological observations of human material, concluded that "this formation [transseptal fiber groups] can be considered a defense mechanism against destruction of the marginal periodontium due to inflammation."[7]

The changes in gingival and transseptal fibers and ground substance described thus far have been primarily the result of gingival injury.

Alterations in fibers and ground substance of the periodontal tissue also have been observed in areas of occlusal stress,[8-10] as well as in oral conditions associated with generalized metabolic imbalances such as diabetes,[11] pregnancy,[12] and hormonal gingivitis.[13]

In view of these observations it is suggested that dynamic changes occur within the attachment apparatus prior to its actual destruction as a result of local and systemic aberrations that have weakened the resistance of the tissues to future local injury and prepared the tissue substrate for further breakdown. One may speculate further that the site of action of these changes varies with the type of irritation. Thus, alterations in the connective tissue above the alveolar crest may be primarily the result of gingival injury. Changes in the attachment apparatus that result from occlusal trauma may occur along the root surfaces in areas of occlusally produced tension and pull. General metabolic imbalances may produce changes throughout the entire attachment apparatus.

Response of the Alveolar Bone to Systemic Stressors

A response of the alveolar bone to both local and systemic insults has been the object of innumerable studies, all of which have demonstrated its existence. However, the responses have varied according to the type of insult. Let us discuss, for example, some of the findings reported from various inanition experiments. Most studies report an osteoporosis of the spongiosa of the alveolar bone,[14-18] with a much lesser effect on the alveolar bone proper.* Similar osteoporotic changes have been reported to follow the induction of abdominal malignancies[19] and various physical stressors, such as prolonged exposure to excessive noise and light[20] (FIGURE 3*a* to *c*). One is thus confronted with the observations that an osteoporosis of the spongiosa of alveolar bone is the more marked response to these insults, while changes in the alveolar bone proper and the periodontal fibers are less pronounced: this behavior pattern suggests that the trabeculate alveolar spongiosa has the greater lability of response to metabolic stressors The rationale for such behavior may be found in the concept that the alveolar bone proper receives stimuli through the functional tensions of the principal fibers and thus responds primarily to these demands,[21] whereas the alveolar spongiosa, not being subject to these functional demands to the same degree, can serve as a storehouse for minerals, proteins, and other nutrients that may be mobilized in time of bodily stress. Similar alveolar responses have also been observed in human material. FIGURE 3*d* demonstrates a section through a human mandible. This specimen was obtained from a mandibulectomy for cancer therapy. It is of interest that this osteoporosis

* That portion of it that surrounds the root and gives attachment to the principal fibers of the periodontal membrane.

tium when subjected to combinations of irritants. The superimposition of various local irritants upon systemically stressed animals has demonstrated a delay in repair, which in turn has induced an over-all increase in destruction.[26,27] For example, nutritional deprivation following gingival injury interfered with

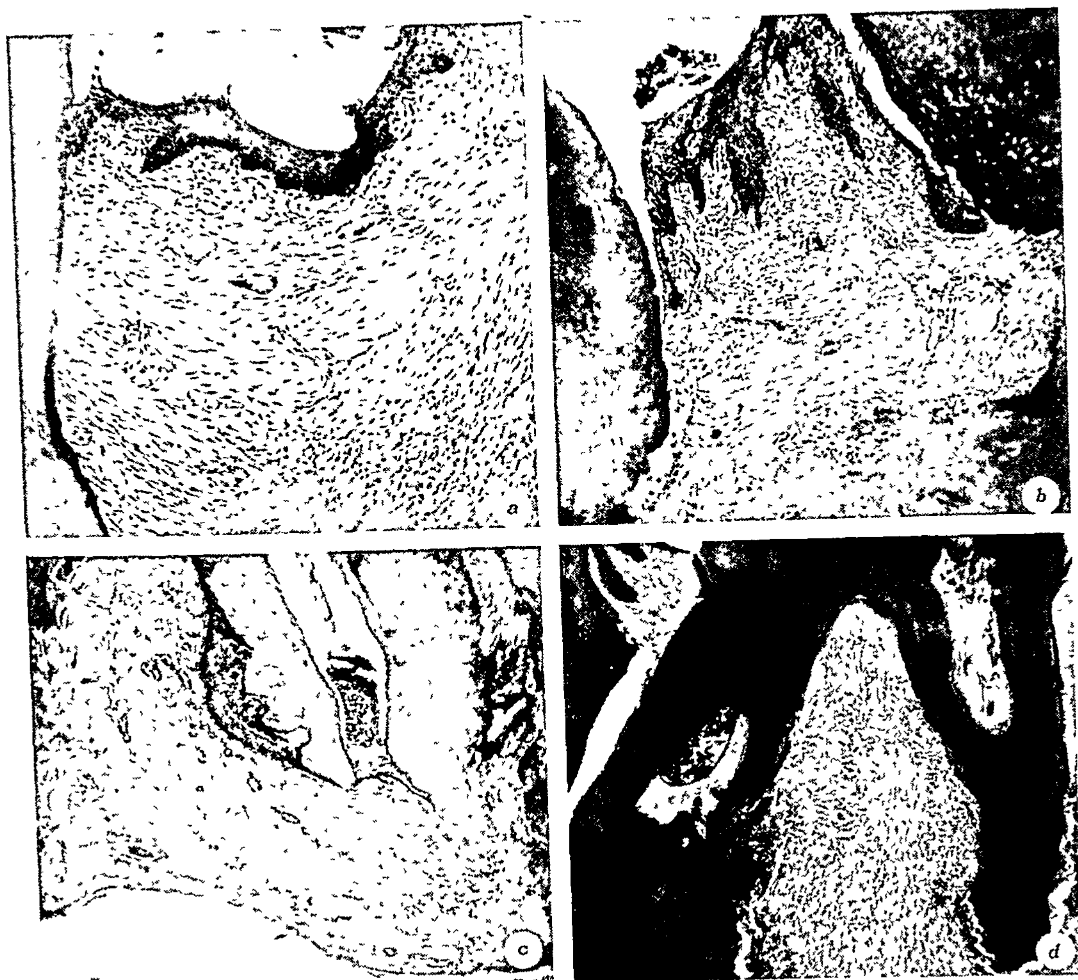


FIGURE 5 (*a*) Rat interdental alveolar crest between first and second molars at site of gingival irritation of 8-weeks' duration [27] Osteogenic repair activity at crest H&E stain; ×100 (*b*) Interdental alveolar crest between first and second molars below injury of rat on protein-free diet for 8 weeks [27] Marked gingival recession with apical proliferation of epithelial attachment H&E stain, ×75 (*c*) Periapical tissues around root of maxillary first rat molar in which a mesial pulpal exposure had been created [28] Osteogenic activity around apex H&E stain, ×45 (*d*) Interradicular area of maxillary first molar of rat that had received both occlusal overloading and pulpal exposure [28] Loss of interradicular septum and its replacement by fibrous connective tissue H&E stain, ×45.

osteogenesis and with new fibroblastic proliferation. In this case, combining a local irritant with a weakened tissue substrate led to a greater destruction of the periodontal supporting tissues than was seen in the control specimens (FIGURE 5*a* and *b*).

Similar observations can be made when occlusal trauma is superimposed upon an apical granuloma resulting from pulpal exposure In our experience, experimental exposure of the pulp of the rat molar caused a circumscribed peri-

marrow cavity. The periodontal structures, on the other hand, were much less affected (FIGURE 4).

The afore-mentioned observations indicate that experimentally induced systemic stressors cause primarily an osteoporosis of the alveolar spongiosa, with some changes in the periodontal fibers, but do not markedly affect the alveolar

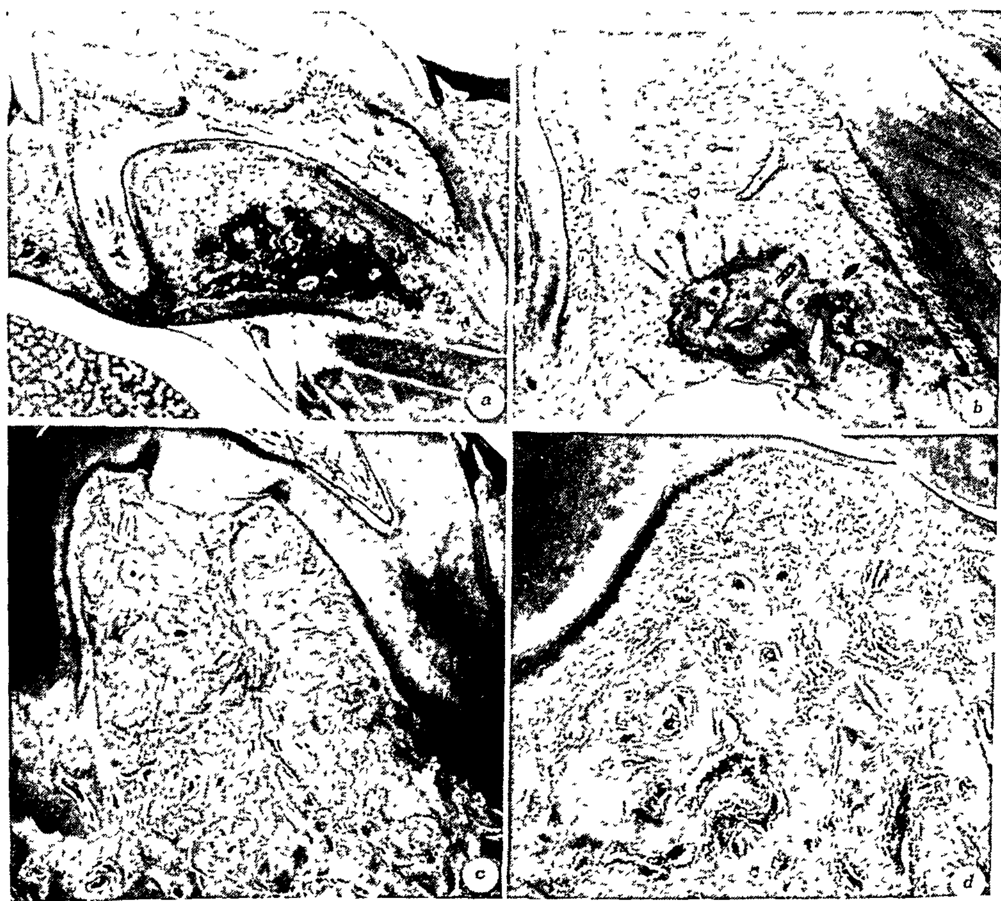


FIGURE 4 (*a*) Interradicular septum of first molar of control mouse. H&E stain, ×42. (*b*) Interradicular septum of first molar of mouse that had received estradiol benzoate injections over an 8-week period[23] Endosteal appostion H&E stain, ×77. (*c*) Interradicular septum of first molar of rat that had received somatotropic hormone injections over a 6-week period[25] Reduction in marrow space with marked endosteal apposition H&E stain, ×40 (*d*) Interradicular alveolar crest of first molar of rat that had received somatotropic hormone injections over a 6-week period[25] Lesser alveolar apposition at crest than marked endosteal apposition shown in *c* H&E stain, ×100

bone proper. Gingival and occlusal injuries, on the other hand, affect primarily the periodontal soft tissues and the contiguous alveolar bone proper.

Response of the Periodontium to Simultaneously Occurring Multiple Insults

The responses of periodontal soft and hard tissues to a variety of local and systemic aberrations acting individually in the experimental design have been discussed thus far. I now propose to deal with the response of the periodon-

greater among people of low educational background than among the relatively highly educated. As a rationale for this finding he lists among other factors a better nutrition in the more highly educated group.

We have seen that the periodontal tissue response to local irritation may be altered detrimentally when an animal is stressed. It is also experimentally possible to alter beneficially the periodontal response of a host by anabolic stimulation. Estrogen, for example, has been shown to reverse the alveolar osteoporosis induced by the administration of cortisone.[32] Similar observations have been made following the simultaneous administration of cortisone and somatotropic hormone.[33] The administration of somatotropic hormone also increased alveolar crest repair in the experimentally wounded gingivae of rats[34] (FIGURE 6*a* and *b*).

Healing of Experimental Gingival Injuries

For the past two years my associates and I have been trying to obtain a repeatable standardized gingival wound in a small experimental animal. The method is at present under study in our laboratory. It was noted that tissue responses to local gingival injury in the rat were similar to those in human beings.[35] Epithelization of the wound occurred prior to regeneration of the alveolar crest. The regeneration of the alveolar crest, however, occurred only when food impaction in the area was absent or kept to a minimum, a fact suggesting that continuous local inflammation interferes with complete repair. Regeneration of the alveolar crest also could be reduced if the animal were placed under generalized stress, which in turn produces partial inanition[36] (FIGURE 6*c* and *d*).

These observations indicate that epithelization of the wound takes place within the normal time limit in the stressed animals, but that the repair of the underlying connective tissue, chiefly crestal osteogenesis, may be greatly delayed.

Summary

From the animal studies described it would appear that a gingival injury initiates the periodontal lesion. Once this is initiated, changes, probably enzymatic in character, occur well below the surface ulceration and alter the stability of the transseptal fiber apparatus. Further inflammatory spread may lead to alveolar crestal resorption.

Systemic stressors, acting individually, induce primarily an alveolar osteoporosis with lesser changes in the fiber attachments, but apparently they do not induce gingival ulceration. However, when systemic stressors are coupled with local irritation, repair of the injury may be delayed. This delayed repair is not seen in a slower epithelization of the wound, but rather in an interference with regeneration of the periodontal fibers and, even more strikingly, with delayed and lesser regeneration of the crestal alveolar bone. It is suggested that the prolonged repair of these vital elements in the tooth attachment apparatus weakens the area in respect to further local irritation and thus enhances the severity of the subsequent lesion.

These experimentally derived definitions of the role of various etiological

apical granuloma to form after about six weeks. The periapical area showed evidence of organization with repair of bone surrounding the root tip. The superimposition of occlusal trauma upon such an exposed tooth, however, interfered with repair, principally by interfering with osteogenesis in the area,

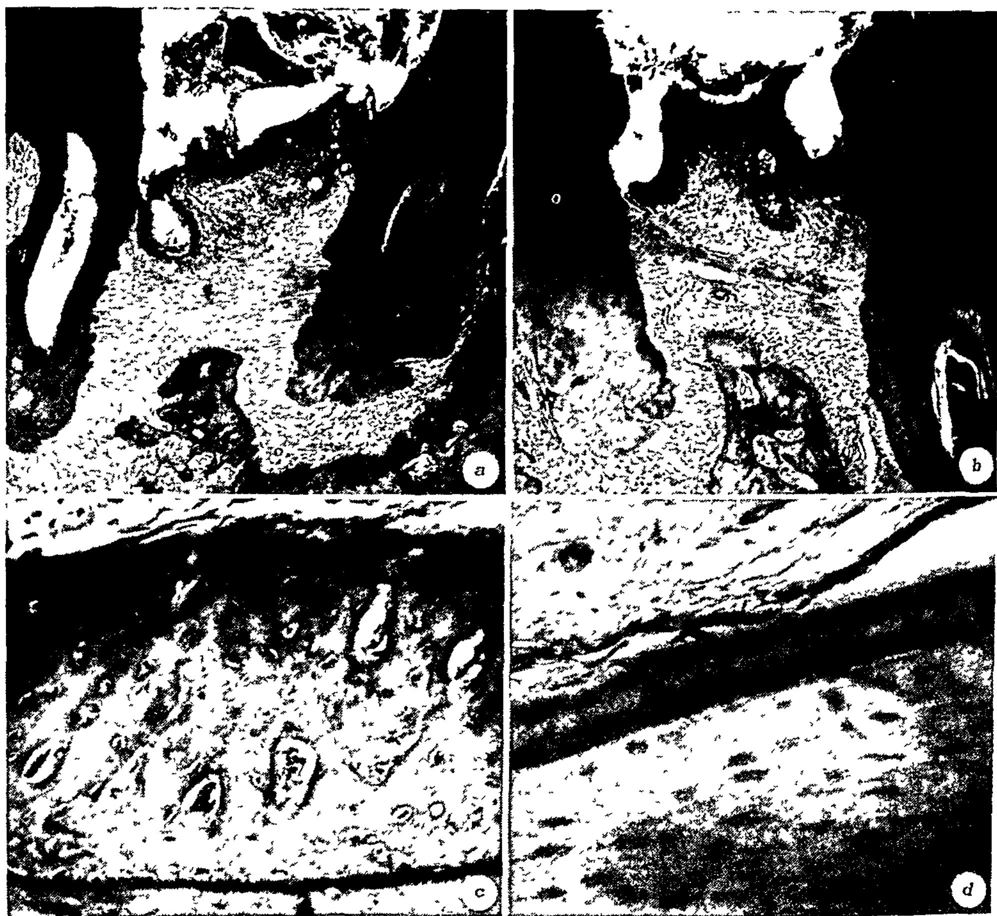


FIGURE 6 (*a*) Injured interproximal papilla of rat that had received injections of vehicle into gingival papilla over 6-week period [34] H&E stain, ×50 (*b*) Injured interproximal papilla of rat that had received injections of somatotropic hormone over 6-week period [34] Crestal osteogenesis H&E stain, ×50 (*c*) Gingival injury created mesially to first molar of a rat Duration of experiment, 24 days [36] Marked osteogenesis along alveolar surface. H&E stain, ×140 (*d*) Gingival injury created mesially to first molar of rat under stress Duration of experiment, 24 days Lesser extent of crestal apposition than in specimen shown in *c* H&E stain; ×140

and this resulted in an almost total replacement of the alveolar septum by fibrous connective tissue[28] (FIGURE 5*c* and *d*).

If it could be demonstrated that such interference with proper repair may also occur in humans, it would increase our understanding of the clinical observation that patients with long-standing chronic illness often exhibit more severe periodontal disease than do healthy patients of the same age [29] Similar observations also have been made in studies correlating social factors with periodontal disease.[30] Russel[31] noted that the incidence of periodontal disease was

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